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THE HOSPITAL OF THE ROCKEFELLER INSTITUTE.

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EXPERIMENTAL STUDY OF THE NASOPHARYNGEAL SECRETIONS FROM INFLUENZA PATIENTS.

Preliminary Report.

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This experimental study of the nasopharyngeal secretions from influenza patients was made during the course of one and a half years in three successive periods. The first period coincided with the epidemic wave of 1918–1919. During this period were studied actual cases of acute uncomplicated influenza and persons who had never been affected. The second period embraced the late autumn of 1919, during which influenza did not prevail in New York in epidemic form. During this interepidemic stage, healthy controls were studied. The third period, during the winter of 1920, saw a return of the epidemic. At this time additional cases of the disease were available for investigation.

By proceeding in this manner we hoped to check our results for each period against one another. As the sequel will show, we believe we succeeded in this undertaking, with the consequence that we are enabled to present our findings with perhaps a degree of confidence not otherwise appropriate.

In planning our experiments we had in mind the possible presence, in the nasopharynx of persons suffering from acute epidemic influenza, of some agent the effects of which might be noted in animals.

In considering the criteria of activity of this agent, we thought in the first place of the well known phenomenon in man of leukocytic depression, involving especially the mononuclear cells, during the acute influenzal infection. In the next place, we had in mind changes of a more or less pronounced but possibly transient character, arising in the lungs, which might conceivably predispose to the severe pneumonias that often accompany as a secondary of concurrent infection the influenzal attack.

The materials with which we worked were the saline washings from the nose and throat. We secured these materials from eight cases of influenza within the first thirty-six hours of the disease, and from twelve cases at later stages, namely, either during convalescence or the period of the postinfluenzal pneumonia. In addition, fourteen persons, during the epidemic or interepidemic periods, believed never to have had influenza were washed in the same manner and their washings studied.

Full grown rabbits were used for inoculation, and no rabbit suffering from snuffles or any detectable disease was employed. All animals were subjected to preliminary blood counting, temperature taking and weighing, and any showing variations beyond the average were discarded. These observations were made on three to seven successive days previous to inoculation.

The inoculations were made directly into the lungs by means of the intratracheal catheter; 3 c.c. of material was the usual dose for a 2.5 to 3 kilogram rabbit, and consisted of (a) the unfiltered nasopharyngeal washings, (b) the filtered washings, (c) lung tissue suspensions, filtered and unfiltered, from previously inoculated rabbits, and (d) similar lung tissue preserved in sterile 50 per cent. glycerin.

It is desirable, in this place, to state that unfiltered washings were employed in the expectation that they could be purified, or rather deprived of their ordinary bacteria, by successive animal passages. It was believed that if this could be accomplished there might be a better chance of preserving and, possibly, bringing to multiplication some other variety of micro-organism, more resistant and virulent perhaps, which would give to the washings from cases of uncomplicated influenza a quality lacking in others. It was, of course, realized that not in every instance could this favorable outcome be looked for. Now and again it was to be expected that a virulent pneumococcus or streptococcus would set up a pneumonia to which the animal would succumb. But if the ordinary bacteria could be suppressed by animal passages in a few instances and something survive which produced definite changes in the structures of the rabbits— in the blood and lungs, for example—the washings from cases of influenza might thus be characterized in a way distinguishing them in effects from the washings of another origin. In this manner the

operation of a usual pathogenic agent is to be deduced, although it might not be possible to determine certainly that this agent is the inciting microbic agent of influenza. However, if a certain correspondence in tissue and other effects can be shown to exist between the person suffering from influenza and the rabbit inoculated with materials originally derived from influenza cases and free from all ordinary bacteria, a probability as to the nature of the pathogenic agent is introduced into the calculation which encourages further investigation along the indicated lines.

There were inoculated into the lungs of rabbits the unfiltered nasopharyngeal secretions from five cases during the first epidemic and three during the second, in the first thirty-six hours of the disease; and from eleven cases during the first epidemic and one during the second in the later stages of the affection.

Results of Experiments.

The following effects were induced by the materials from seven of the eight fresh cases, but not by any of those from the twelve cases examined after thirty-six hours.

Clinical Effects.—From twenty-four to forty-eight hours after inoculation, fever developed, associated with the ordinary signs of indisposition in a rabbit, such as listlessness and ruffled hair. This was accompanied by conjunctivitis, varying from injection to pronounced catarrhal inflammation. The striking feature, however, was the definite and often marked leukopenia resulting from the depression of the mononuclear cells, as shown in the accompanying chart. If the condition was allowed to run its natural course, these symptoms endured for three days, the animal then returning to normal. If the rabbit was killed—for if the condition remained uncomplicated by ordinary bacterial infection none died—an unusual pathologic picture was revealed.

Pathologic Effects.—The respiratory organs were affected to the exclusion of all other structures. No pleuritis or exudate in the pleural cavity was evident. The lungs were voluminous and·edematous, and had a mottled, hemorrhagic appearance. The hemorrhages on the surface, beneath the pleura, were diffuse or discrete, occupying

areas of a few millimeters in extent, or covering a large part of a lobe. In addition, minute petechiae occurred. On section of the lungs the cut surface revealed a hemorrhage edema; it dripped a blood-stained, frothy fluid. The hemorrhages again were either diffuse and large or discrete and small; in the latter instance they were numerous.

Microscopic sections of the lungs were made through various parts, the base, periphery or hilum, and as a rule were carried through the hemorrhagic foci. The hemorrhages were either diffuse, invading large areas of pulmonary structure, or were localized to small areas, or seen as extravasations into the interalveolar and 'intra-alveolar structures. The edema was pronounced: the alveoli were filled with serum, and there was a serous exudation in the interalveolar strands. The lung structure showed a cellular exudate, comprising polymorpho-nuclear cells showing usually large eosinophilic granules; large cells of an alveolar type, probably desquamated bronchial epithelium, and in the interalveolar strands, mononuclear cells. Some fibrin was present as well. The bronchi were partly filled with erythrocytes, fragments of degenerated and exfoliated epithelia, and leukocytes. Their walls here and there had lost their epithelial lining, and were hyperemic and thickened. The capillaries were distended with blood.

No ordinary bacteria were seen in impression films of the lung tissue or in sections stained by Gram's or MacCallum's method, or in cultures of the tissue.

If the clinical effects observed continued for forty-eight hours, the animal as a rule was killed, and the affected lung tissue was ground, suspended in saline solution, and injected into the lungs of a new series of rabbits. In this way the clinical and pathologic effects were passed through as many as fifteen successive rabbits.

These effects were obtained only with the nasopharyngeal secretion in seven of eight patients, collected within thirty-six hours after the onset of the first symptoms. Twelve cases of influenza, in which the washings were collected forty-eight hours after onset and in the stage of convalescence or of secondary pneumonia, failed to give the same results.

Control experiments were made by injecting into the lungs of rabbits saline solution, suspensions of normal rabbit lungs, normal rabbit serum, foreign protein, such as human ascitic fluid, bacteria

of the ordinary species, including Pfeiffer's bacillus and its poison as prepared by Parker's method, and finally the nasopharyngeal secretions from fourteen persons free from influenza and tested in the epidemic and interepidemic periods Of the latter, seven suffered from early or later stages of coryza None of the animals inoculated
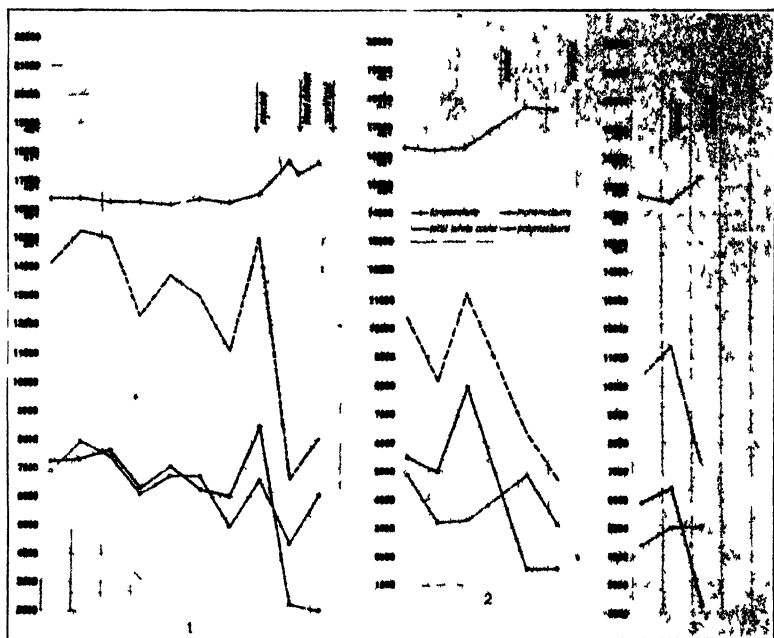
Chart illustrating effect on blood count To be noted are the rise in temperature and the depression in the total white blood cell count caused by a deficiency of mononuclears 1, first rabbit passage of the nasopharyngeal washings from a fresh case of uncomplicated influenza, also the normal curve of this animal prior to injection; 2, second rabbit passage, 3, seventh rabbit passage.

with the control materials or these secretions showed the same clinical and pathologic action, a few gave a polynucleosis with frank lobar pneumonia; others, a mononucleosis without visible lung involvement and still others, inconstant effects

A closer study was then made of the agent causing these phenomena, and it was found that although it was extremely difficult to

secure these results with filtrates obtained directly from the patient's secretions, they could be obtained by first passing the material through a series of rabbits. Then repeated filtration through Berkefeld "V" and "N" candles between rabbit passages did not hinder the development of the typical clinical and pathologic effects.

We determined that the agent was able to resist the action of sterile 50 per cent. glycerin for nine months, but probably not for a much longer period. In one series of experiments glycerinated material free from ordinary bacteria was passed with typical effects through ten successive rabbits.

No case of the uncomplicated effect in the rabbits terminated fatally. By "uncomplicated" we mean that cultures of lung tissue after the sacrifice and of blood during the illness yielded no growth of ordinary bacteria.

But although these effects were produced repeatedly in rabbits with material free from ordinary aerobic bacteria, the latter micro-organisms were encountered occasionally in the course of the experiments.

Those animals in which the inoculation terminated fatally did yield growths of various bacteria from the lung tissue, thus: pneumococcus, Group IV, eleven times; atypical II, three times; *M. catarrhalis*, a gram-negative hemoglobinophilic organism resembling Pfeiffer's bacillus, and *B. pyocyaneus*, each twice; *Streptococcus viridans*, streptothrix, and *B. coli-communis*, each once. Generally the same organism was not found regularly in the consecutive rabbit passages of a series of transmissions. For example, in the filtrate series of Case 17 the fifth passage showed a pneumococcus Group IV, the seventh and eighth *B. pyocyaneus*, and the tenth a gram-negative, hemoglobinophilic micro-organism, resembling Pfeiffer's bacillus. The other passages were free from such bacteria. In three of the seven series of transmissions, the ordinary bacteria in the secretions were suppressed completely, and all the animal passages remained free from them.

The pathologic picture when complicated by these ordinary bacteria showed severe, extensive lobar or bronchopneumonia with fibrinous and exudative pleurites and abscess formation and, as a rule, a rapid decline and death of the animal.

A series of investigations was undertaken in which an effort was made to reproduce the last mentioned conditions experimentally. Rabbits were injected with the influenzal material intratracheally and later with small nonpathogenic doses of the pneumococcus, Pfeiffer's bacillus, and *Streptococcus viridans* intravenously. In these animals the bacteria localized themselves in the lungs, producing extensive lobar pneumonias or bronchopneumonias with pleuritis and, as a rule, leading to death. Hence apparently this material obtained from the nasopharyngeal secretions in early influenza diminishes the resistance of the rabbit's pulmonary structures against invasion by these common micro-organisms.

<div align="center">CONCLUSIONS.</div>

From the evidence obtained in this study, extending over one and a half years, it would appear that there occurs a specific substance in the nasopharyngeal secretions in cases of epidemic uncomplicated influenza. This substance seems to be present only in the early hours of the disease. It has not been found later than thirty-six hours after the onset, nor in cases of secondary pneumonia, nor in secretions from persons free from the syndrome of influenza either during the epidemic or during nonepidemic periods.

With this substance we have induced a clinical and pathologic condition in rabbits, affecting the blood and pulmonary structures mainly, which could be maintained and carried through at least fifteen successive animals. For this reason, and also because of the dilution between passages and the shortening of the incubation period from rabbit to rabbit, we are led to believe that we are dealing with the actual transmission of a multiplying agent rather than with a passive transference of any original active substance.

This active substance is filterable, and resists the action of sterile 50 per cent. glycerin for nine months, but probably not for a much longer period.

The manner in which the bacteria of ordinary species, such as the pneumococcus, the Pfeiffer bacillus, *Streptococcus viridans* and others are encountered during the course of the transmission experiments and during the experimental reproduction of the condition described

justifies the opinion that these micro-organisms are secondary in effect. The essential effects are produced by a substance wholly unrelated to these bacteria.

The similarity that exists between these effects in rabbits and those occurring in man lays a basis for further investigation on the inciting agent of influenza.

It may be stated here that during the course of these experiments we have seen in cultures, both from the lung tissue of affected rabbits and in the filtered nasopharyngeal washings from cases of influenza, tiny bodies, almost invisible, which decolorize by Gram's method and which stain generally with difficulty with nuclear dyes. This phase of the subject is still under investigation.

EXPERIMENTAL SYPHILIS IN THE RABBIT.

II. Primary Infection in the Scrotum.
Part 1. Reaction to Infection.

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PLATES 72 TO 82.

Primary skin lesions in the rabbit were first reported in 1908. Hoffmann, Löhe, and Mulzer (1), and Ossola (2) reported instances of scrotal infection resulting from testicular inoculation, while Levaditi and Yamanouchi (3) succeeded in producing infection by inoculation of the prepuce. With the introduction of skin inoculations, a decided advantage was gained in the study of experimental syphilis. Not only were the conditions of the inoculation more akin to the mode of infection in the human subject, but the lesions themselves were more analogous to the primary lesions of man. In addition, the infection took place in a region more accessible to observation and the reaction to inoculation could be followed directly, which in itself was a decided advantage.

Of the various skin areas in which inoculation was attempted, the scrotum proved to be best adapted and is now the only area used for purposes of routine inoculation. The methods devised for scrotal inoculation were of three types, all of which were quite simple and consisted in the implantation of bits of infected tissue beneath the skin of the scrotum, in the injection of a fluid medium containing the infecting organisms, or in scarification of a skin area and the local application of the virus. Satisfactory results have been reported from the use of all three of these methods.

EXPERIMENTAL.

In our own work upon scrotal infections of *Treponema pallidum*, the experimental conditions employed were much the same as those outlined in the case of the testicular infections (4).

Animals Used.—A great variety of animals was used, but those preferred were young albinos, grays, browns, or Dutch belts. Individual animals were chosen in which the scrotum was thin and delicate and as free from hair as possible. Old animals with thick, fibrous, or redundant scrota gave results inferior to those of younger animals with more active tissue.

Method of Inoculation.—Inoculation of the scrotum was carried out according to one of two methods. The procedure commonly employed was essentially the same as that described by Tomasczewski (5) which consists in the implantation of a small piece of infected tissue in a pouch prepared for it in the scrotum. The tissue used was either an infected testicle or a granulomatous skin nodule removed with as little contamination as possible and cut into pieces measuring from 3 to 5 mm. in diameter. The inoculation was performed either by the use of a trocar needle of 8 to 9 gauge inside diameter or by snipping the superficial layers of the scrotum with scissors and introducing the fragment of tissue by means of a pair of forceps; the latter method was in general preferred. The only precautions necessary were the observance of reasonable cleanliness and making the implants as superficial as possible.

Both of these methods proved highly satisfactory when rapid development of large skin lesions was desired. They are not adapted, however, to the study of the finer details of the reaction to infection, and ·for this purpose, we used an intracutaneous or subcutaneous injection of a virus emulsion prepared as for testicular inoculation except that the emulsion contained a greater number of spirochetes, averaging 5 to 10 to the microscopic field. In this operation, the scrotum was cleansed as usual and 0.1 to 0.2 cc. of the emulsion injected by inserting the needle well above the point to be inoculated and carrying it downward to the desired location.

Material Studied.—During the past 4 years, we have inoculated a large number of rabbits by these two methods. Among the earlier animals, there was an occasional one in which infection could not be established with·absolute certainty, but all animals inoculated during the last 3 years have developed characteristic lesions from which *Treponema pallidum* could be recovered.

The material afforded by these rabbits was used in various ways. After well developed lesions had become established, a majority of them were used for therapeutic experiments and hence were not available for further study of the course of the local infection except where recurrent lesions developed. Some lesions were excised for histological study at various stages of their development, but a large number of rabbits was held under constant observation throughout the course of the local infection,—some of them as long as 18 months and a few for 2 years or longer.

Reaction Following Inoculation with a Virus Emulsion.

By whatever method scrotal inoculation is performed, the specific reaction which takes place is essentially the same. The early stages of the reaction can be traced more accurately, however, by the use of some methods than by others, and for this purpose, we used an emulsified virus which is but little more than a diluted tissue extract containing the *pallidum*. When inoculations are properly performed by this method, there is very little traumatism, and the slight inflammatory reaction which may develop on this account disappears completely within 24 to 48 hours. Thus, one is not only able to keep the dose of virus used comparatively uniform and to control the conditions of inoculation, but the resulting reaction is as little complicated by extraneous circumstances as is practicable upon any large scale. The chief objection to the method is that it tends to diffuse the infection over a wider area and the resulting reaction is not always so sharply circumscribed as one might wish.

Incubation Period.

The incubation period of scrotal infections produced by inoculation with a virus emulsion came within the comparatively narrow limits of 1 and 4 weeks. Under favorable circumstances, a definite reaction was usually recognized within 10 days to 2 weeks after inoculation and in many instances by the end of the 1st week. Occasionally, the specific reaction developed more slowly and characteristic lesions were not recognizable for several weeks.

The average incubation period of scrotal infections was somewhat shorter than that previously given for the development of gross or clinical alterations in the testicle but corresponded fairly well with

the time within which lesions could be demonstrated microscopically or within which multiplication of spirochetes could be determined by dark-field examination of fluid obtained from the testicles.

Form of the Initial Reaction.

The specific reaction in the scrotum appeared in one of three general forms differing somewhat according to the location of the lesion. The first and most important form of reaction began as small circumscribed swellings or diffusely spreading patches of a rose-pink color situated in the papillary and reticular layers of the skin. These lesions were soft or gelatinous in character and were frequently associated with a rich vascular network such as that shown in Fig. 1, which is probably the most significant feature of the syphilitic reaction. The second form taken by the initial lesion was that of a minute translucent nodule or plaque of induration likewise situated in the papillary layer of the skin but more superficial than the first (Fig. 5). The third type of lesion appeared as an opaque porcelain-white nodule or plaque of extreme hardness situated in the depths of the scrotum and was usually connected with the outer surface of the tunica vaginalis. The first two forms of reaction were the most common and usually the earliest to appear.

Development of the Primary Lesions, or Chancres.

The development of primary skin lesions, or chancres, from initial foci of reaction, such as those described, followed a course which may be represented in general by Figs. 1 to 4 and 5 to 8 which were from two animals of the same series. As indicated in these illustrations, the progress of the infection was marked by an extension of the reaction and the occurrence of certain transformations. The infection tended to spread diffusely through the scrotum, and as the reaction about the focus of infection increased, lesions were formed which assumed the character of circumscribed nodular elevations or of flattened, diffusely spreading patches.

In the development of the gelatinous type of lesion (Figs. 1 to 4), the swelling first subsided to a slight extent and the color changed to a more coppery tint. The lesions then became firmer, and as the

induration increased, changed to more opaque, porcelain-white masses. Purplish red spots and streaks of congestion and hemorrhage then appeared over the central portions of these lesions and with them yellowish gray or yellowish brown areas of necrosis which tended to spread and become covered with thin scales or crusts. Finally, the central area of the lesion became necrotic and sloughed away or was covered by a more or less continuous crust.

In the case of circumscribed lesions such as those in Figs. 1 to 4, these changes led to the formation of elevated masses with a necrotic or ulcerated center surrounded by a zone or collar of induration. If the lesions spread diffusely, as they not infrequently did, the resulting lesion was a thickened or indurated patch over which the areas of necrosis were more diffusely scattered; the crusts were usually imperfectly developed and loosely attached, and while ulceration was slight, weeping patches were formed here and there.

When the scrotal lesions first appeared as translucent nodules or patches of induration, development of the lesions followed a slightly different course (Figs. 5 to 8). The vascular reaction about such areas was relatively less than in the preceding case. As the lesions increased in size, the central portions became more dense and opaque, while the overlying skin became smooth and glistening. The covering epithelium was thinned out until small defects or superficial areas of necrosis appeared and were covered by thin scabs or crusts.

When these lesions assumed the form of circumscribed nodules (Figs. 5 to 8), the area of necrosis enlarged and deepened with the growth of the lesion and was covered by a thick crust or formed a depressed ulcer surrounded by a mass of indurated tissue. The more diffuse lesions of this class appeared as parchment-like thickenings in the skin which either remained intact and appeared translucent, smooth, and glistening throughout or were irregularly covered by loosely attached scales with raw or ulcerated areas here and there.[1]

The third form taken by the initial lesion in the scrotum, that of an opaque white nodule or plaque of induration in the depths of the scrotum, usually followed a still different course of development.

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[1] See section below on diffuse scrotal lesions.

As in the case of the lesions just described, the vascular reaction about these lesions was of minor degree. They developed rather slowly as a rule and grew or spread in the form of extremely hard masses over which the skin was freely movable. The plaques usually remained deep seated and spread along the surface of the tunica vaginalis, but the nodular lesions gradually extended towards the surface so that the overlying skin not infrequently became involved. Necrosis and ulceration then took place with the formation of lesions much like those which have been described. However, many of these deep seated nodules did not involve the overlying tissues to a sufficient extent to bring about necrosis and ulceration but remained throughout as circumscribed nodular lesions in the deeper tissues of the scrotum.

The subsequent course of the reaction to infections produced in this way was essentially the same as that of an infection produced by other methods of inoculation and may be followed out in connection with the infection produced by implantation.

Reaction Following Inoculation by Tissue Implantation.

While inoculation by the use of tissue implants possesses certain advantages, there may be some difficulties attending its use. Due to unequal distribution of spirochetes in infected tissues as well as to inequalities in the vitality of the organisms present in different portions of a lesion, inoculations carried out by implantation may not yield results which are as uniform as from the use of methods by which the dose of virus can be kept more nearly constant. It is possible, however, to overcome these difficulties to a large extent by the observance of certain precautions: first, the use of infected tissues at a relatively early stage of the infection or only at a period when the infection is actively progressing; second, the use of only such portions of a lesion as are actively growing at the time; and third, as a means of insuring an adequate dose of organisms when any doubt exists, the use of pieces of tissue as large as can conveniently be used without causing necrosis and sloughing of the surrounding tissues. Obviously, judgment in the use of these precautions can be acquired only by experience. A second difficulty in the

use of this method is the traumatism necessarily inflicted and the chances offered for the development of secondary infections. These need not prove serious, however, since reasonable care in the performance of inoculations will remove such difficulties to a considerable extent.

For certain purposes, implantation has advantages over other methods of inoculation which more than compensate for its disadvantages. When properly safeguarded, large, actively growing skin lesions can be obtained more quickly and with greater certainty by this method than by any other method of inoculation with which we are acquainted.

Incubation Period.

The exact incubation period of infections produced by scrotal implantation is difficult to fix since a non-specific foreign body reaction frequently overshadows the specific reaction. As nearly as can be determined, however, the incubation period in our series of animals fell within much the same limits as for inoculations made with emulsions. There was usually a definite specific reaction about the implant, and spirochetes could be obtained from the surrounding tissues within 10 days to 2 weeks after inoculation and were obtained as early as the 3rd day, but it was not certain that the organisms found were more than transient invaders. In a few instances, incubation was unusually prolonged after inoculations performed by this method, and no specific reaction could be recognized for several months—in one instance 6 months and 6 days. This unusual prolongation of the incubation period probably had no connection with the method of inoculation used except as a possible instance of the inoculation of a small number of organisms or of organisms of low vitality, and is merely cited to show the time which may elapse before any definite reaction can be detected.

Early Reaction.

The reaction which takes place in the scrotum following inoculation by implantation may be regarded as partly one of wound healing, partly a foreign body reaction, and partly specific in character. During the first 24 hours, the scrotum became slightly reddened and

edematous, but this acute inflammatory reaction rarely lasted beyond the 2nd or 3rd day.   Then the process of organization became established; the swelling subsided, leaving the skin about the implant slightly reddened, smooth, and glistening.   This condition persisted for upwards of 7 to 9 days without producing any considerable increase in the size of the nodule.   In some instances, the skin over the implant became necrotic and was covered by a crust or sloughed away leaving a depressed ulcer surrounded by a zone of granulation tissue.   If no specific reaction had developed by this time, the nodule began to shrink and soften while the skin became relaxed as the process of organization or healing subsided.   If the tissues about the implant retained their appearance of activity beyond the 10 day period, and especially if the reaction appeared to be increasing, it was usually found that a specific reaction had commenced.

However, the specific reaction usually became apparent after the process of organization had abated to an appreciable extent and appeared almost as a renewal of this process.   The differences which could be recognized were that there was less reddening in the specific reaction, the tissues were more translucent and more definitely indurated, and the reaction resulted in a rapid growth of the lesions. In many instances, there was no break in the continuity of the two processes and it was impossible to determine exactly when the specific reaction began.

Not infrequently, multiple foci of infection resulted from inoculation by this method.   Thus, lesions developed independently about the implant at the point of incision and occasionally other discrete foci were scattered along the track of the sinus.

Course of the Scrotal Reaction and Development of Typical Scrotal Chancres.

In following out the successive stages in the scrotal reaction and the development of what may be called typical scrotal chancres, it may be assumed that the specific element in the reaction is the same in all essential respects whether inoculation is carried out by the use of a virus emulsion or by tissue implantation.   The mechanical conditions of the reaction in the two cases are quite different, however.

The production of an infection by the introduction of a solid mass of tissue into the scrotum undoubtedly exercises a considerable influence in itself upon the character of the lesions produced and favors the development of large circumscribed lesions, but chancres produced in this way do not always follow the same course of development, and the resulting lesions themselves may be quite different. It is well, therefore, to consider the growth of these lesions according to the general character of the reaction.

Three characteristic examples of chancre development following the use of this mode of inoculation are given in Figs. 9 to 12, 13 to 16, and 17 to 20. The first group of photographs (Figs. 9 to 12) represents stages of chancre development where optimum conditions of virus, animals, and technique were combined. The animal shown was one of a series of ten rabbits in which the results were remarkably uniform. The first photograph (Fig. 9) taken 10 days after inoculation shows a well established specific reaction. There were some edema and congestion of the scrotum, and the skin over the implants had undergone necrosis with the formation of hard dry crusts which showed a line of demarcation at their periphery. Both implants were surrounded by a distinct but narrow zone of specific granulation, more prominent on the left than on the right. These lesions developed very rapidly and by the 13th day (Fig. 10) presented all the characteristics of typical chancres with depressed ulcers surrounded by broad zones of induration.

With the growth of the lesions as illustrated in Figs. 11 and 12, taken 3 and 5 weeks respectively after inoculation, the diffuse congestion and edema of the scrotum persisted; the scrotum gradually became thickened and a few petechial hemorrhages appeared towards the lower end of the right scrotum (Fig. 11), which formed the center about which a second circumscribed lesion soon developed. Eventually, the entire scrotum of both testicles became involved in the specific reaction and the condition existing at the end of the 5th week was that shown in Fig. 12. On the right, there were two circumscribed lesions connected by areas of more diffuse infiltration, while on the left, there was a single massive chancre surrounded by tissues which were themselves markedly infiltrated and covered by diffusely spreading patches of hemorrhage and necrosis.

This is an instance of a high grade infection in the scrotum of the rabbit such as may be produced under favorable conditions and has been observed many times. It may not be out of place to point out that this case illustrates, in particular, first the extreme rapidity with which the infection may become established and typical circumscribed lesions developed, and secondly the inherent tendency of

pallidum infections to spread beyond the local confines of the initial focus of infection, together with something of the character of the reaction which occurs under such circumstances.

The second case in this group (Figs. 13 to 16) illustrates a course of chancre development which is more nearly an average than that just described. The reaction exhibited by this animal was of the same general character as that of the first, but differed in two essential respects. There was early ulceration of the lesions as in the former case, and by the 14th day, characteristic chancres with well defined collars of induration were present (Fig. 13). The growth of the lesions then ceased, the edema in the scrotum subsided, and the chancres themselves decreased in size (Fig. 14). During the 4th week, there were a slight recurrence of the edema (Fig. 15) and renewed growth of the circumscribed lesions which continued with slight remissions until large indurated lesions were formed (Fig. 16, 57 days after inoculation).

These two examples will serve to illustrate the type of reaction which takes place in most instances of active chancre development. A third form of reaction commonly seen is that illustrated in Figs. 17 to 20. This animal was one with a rather thick fibrous scrotum (note the scrotum in Figs. 17 and 18) and the implants could not be made so superficially as one would wish. The incubation period was somewhat longer, and the lesions did not begin to grow actively until about the end of the 4th week after inoculation. The photograph reproduced in Fig. 17 was taken at the end of the 5th week (34 days) when the nodules measured approximately 1 cm. in diameter. The skin about the nodules was still but little affected and for the most part retained its normal appearance. During the next 18 days, these lesions underwent a considerable transformation (Figs. 18 to 20); they increased in size and became more indurated, while the process extended to the overlying skin which became smooth, translucent, and highly refractile. Areas of necrosis then appeared upon the surface, and as these extended and contracted, the lesions flattened out into the form seen in Fig. 20. Specific reactions of this type were especially apt to occur when the lesions developed in the depths of the scrotum or when, for any reason, the reaction pursued a less active course and involvement of the skin with consequent necrosis and ulceration took place at a relatively late period in the growth of the lesions.

Multiple Chancres.—Multiple lesions of a chancre-like character developed in the scrotum under two different conditions, first as a result of simultaneous infection of several points in the scrotum, and second as a result of spread of the infection from a given focus. While lesions of both classes might be regarded as chancres, the significance of the two cases is different, and in the present connection, we shall refer only to lesions of the first class.

Inoculation of the scrotum of the rabbit by the methods described usually resulted in the production of unicentric lesions. Not infrequently, however, lesions appeared simultaneously at other points reached directly in the process of inoculation. The most common seat of the accessory chancre was the point of incision in the skin, and a typical instance of an early lesion of this kind is shown in Fig. 21, the photograph of which was taken 14 days after inoculation. Accessory lesions of this type were frequently abortive or were overgrown by the more vigorous reaction about the main focus of infection as was the case in this animal (Fig. 22, 36 days after inoculation).

A second case of a similar character is shown in Fig. 23. Originally there was an accessory focus of reaction on both sides of this animal. That in the right scrotum was abortive and had almost disappeared when this photograph was taken (29 days after inoculation), while the one on the left was growing quite actively.

In some animals the accessory chancres reach a considerable size even though they are encroached upon by the main chancre mass. Figs. 24 and 25 show a case of multiple chancres in which there were three foci of reaction in the right scrotum and two in the left (Fig. 24, 29 days after inoculation). As these lesions developed, the middle focus of infection on the right became obliterated, but the other accessory chancre grew almost as vigorously as the main lesion. On the left, the two lesions fused at their proximal borders forming a figure of eight chancre (Fig. 25, 49 days after inoculation).

In rare instances, one finds little difference in the vigor displayed by the several lesions present, and all may develop at about an equal rate as shown in Fig. 26 (47 days after inoculation). In addition to the group of multiple lesions described, all of which were situated in the skin itself or pointed upon the skin surface, there was another interesting group of multiple focal lesions in which the foci of reaction were located at different levels in the scrotum—one above the other; these lesions were situated in the skin and upon the outer surface of the tunica vaginalis. This form of localization occurred especially after subcutaneous inoculation with a virus emulsion and is of especial interest in revealing a tendency of *Treponema pallidum* to localize in these two structures.

These few examples of the reaction to infection in the scrotum and the growth of the primary lesions will serve to illustrate what may be called typical skin reactions and typical chancre development in the sense that they represent processes which are in themselves perfectly characteristic and result in the formation of lesions which are easily recognized as lesions of a specific character. In many instances, however, reactions occur and lesions develop which are not so well recognized as processes of a syphilitic nature, and we may refer briefly to a few instances of this·kind.

Influence of Different Elements of the Specific Reaction upon Chancre Development.

Irregularities of Chancre Development.

The condition which gives the characteristic picture to the specific reaction in the skin and to the chancre itself is the maintenance of a certain balance among the several processes which take part in this reaction.   It was found that when for any reason this balance was disturbed, the character of the reaction was altered and the lesions became modified in accordance with existing conditions.   The irregularities of chancre development which were most commonly seen were associated with the occurrence of excessive edema or of congestion and edema, with conditions simulating an acute inflammatory reaction, or with irregularities in the process of granulation, the cause of which cannot be discussed.

Edema.—It was found that excessive edema with or without congestion might occur at any time during the course of the local infection.   When it developed at an early stage of the infection, it not infrequently overshadowed the proliferative reaction and produced a lesion which showed chiefly a depressed ulcer with a firm margin surrounded by an edematous skin (Fig. 27).   In some instances, this condition persisted for some time, while in others it was no more than an important feature of the reaction or an event in the course of the reaction.   Figs. 28 and 29 illustrate an instance of this kind in which the specific reaction was proceeding rather slowly, and at the end of the 3rd week, there were ulcers surrounded by a narrow zone of induration.   An acute edema then developed (Fig. 28), and with the appearance of this edema, the focal lesions began to grow at an extremely rapid rate; in 2 weeks, they had reached the condition shown in Fig. 29, the edema persisting to an appreciable extent all the while.

Late edema or edema occurring after characteristic lesions had developed was also seen occasionally and was so marked in some instances as to obscure completely the character of the lesion (Fig. 30).

Acute Inflammatory Reactions.—A small number of rabbits in the series showed an early reaction resembling an acute inflammatory process.   There was the usual inflammatory reaction during the first few days following inoculation, and as this subsided, an extremely active process of granulation set in; the tissues surrounding the implant remained reddened or cyanotic and were quite firm, while the zone of induration increased rapidly.   The center of the nodule became necrotic and sloughed, leaving a moist, depressed ulcer, or, if no slough occurred, the necrotic tissue softened to a creamy semifluid mass covered by a crust.   As the reaction progressed, the tissues surrounding the zone of granulation or ev

the entire scrotum became congested and edematous (Fig. 31). This condition usually lasted for not more than 3 to 5 weeks before regression set in. The lesions which appeared subsequently were usually of a minor character, but in some instances, typical chancres were formed as in the case illustrated (Figs. 31 and 32).

At first we were inclined to regard these acute reactions as non-specific, inflammatory processes, but investigation showed that this was not entirely correct. Spirochetes were present in considerable numbers both in the zone of granulation and in the edematous portions of the scrotum entirely removed from the region of the implant, but secondary infection could not be excluded as a factor in the reaction. However, true suppuration did not occur as it usually did where pyogenic organisms were present to any considerable extent. It was noted that these reactions were more frequent when large implants were made from testicles during an early stage of extremely active infections. It seemed possible, therefore, that these were cases of acute specific reaction influenced to a greater or less extent by bacterial infection as well as by the implant itself.[3]

Irregularities in the Process of Granulation.—In many instances, irregularities in the development of scrotal chancres were traceable to peculiarities in the process of granulation, and since this is such a noticeable feature of what we are accustomed to regard as the typical *pallidum* reaction, the peculiarities naturally took the form of a lowered intensity of reaction or of some irregularity in the process, both of which were commonly seen in scrotal infections in the rabbit.

The case illustrated in Figs. 33 to 36 furnishes an example of an abortive skin reaction with subsequent development of granulomatous nodules in the subcutaneous tissues of the scrotum. The lesions first formed were simple ulcers surrounded by a thin, parchment-like zone of induration in which spirochetes were present (Fig. 33, 35 days after inoculation). On the left, the zone of induration increased slowly and a characteristic skin chancre was produced (Fig. 34, 83 days after inoculation). Meanwhile, the skin lesion on the right had regressed, and a small nodule had developed in the subcutaneous tissues (Fig. 34). Then followed a period during which the lesion on the left extended into the subcutaneous tissues, while the induration in the skin diminished and the ulcer healed (Figs. 35 and 36, 139 and 188 days after inoculation).

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[3] In this connection, it may not be out of place to call attention to the fact that in all syphilitic infections, the presence of a toxin as an exciting factor distinct from the organisms must be kept in mind. We have avoided introducing this element into the discussions, since we are not prepared, as yet, to make any definite statement upon this point. It seems not unlikely, however, that certain features of the syphilitic reaction may find their explanation in the action of such a toxin, and this applies to reactions of the type just described.

A second case of even less characteristic chancre formation is shown in Figs. 37 to 40. The initial skin lesion in this animal was no more than a thickened patch, the surface of which was smooth and glistening with small erosions here and there; some of these were covered by scales, while others were of a weeping character. These lesions underwent many transformations (Figs. 37 to 40), but never developed beyond the point shown in Fig. 37 which represents the condition existing 36 days after inoculation. Here again, the granulomatous lesions which ultimately developed were subcutaneous in origin and were of very slow and irregular growth (Figs. 38, 39, and 40; 92, 106, and 127 days after inoculation).

Diffuse Scrotal Reactions and Transformations of Diffuse Lesions.

As we have already pointed out, there is a tendency on the part of *pallidum* infections to spread beyond the point of inoculation, and not infrequently this local extension of the infection gives rise to lesions of a more diffuse character than those which have been described. No sharp line of distinction could be drawn between these two groups of lesions; it appeared that one was but a step removed from the other, and diffuse and circumscribed processes were frequently coexistent, or one type of lesion might be transformed into the other, instances of which have already been noted.

As a connecting link between these two forms of scrotal reaction, attention may be called to the lesions shown in Figs. 41 to 44. The photograph reproduced in Fig. 41 was taken 33 days after inoculation. In this animal, there was a moderate but fairly well defined thickening in the skin about the incisions as well as about the implants, and the characteristic feature of the lesions was the spreading necrosis in the skin over the implants and the formation of loosely attached scales, or exfoliation, over all affected parts of the skin, which is shown very well in the left scrotum (Fig. 41).

The second photograph (Fig. 42) shows a later stage of a similar lesion. In this animal, there are two things to be noted, first the puckered scar-like areas on both sides, and second the peculiar appearance of the skin about these areas. The skin was in general of a parchment-like character, very smooth and translucent with grayish yellow or yellowish brown scales or crusts distributed over its surface. Needless to say, these portions of the scrotum contained spirochetes in abundance.

The other two figures on this plate (Figs. 43 and 44) illustrate a somewhat different manifestation of the syphilitic infection. In Fig. 43, there is shown a rather irregular and slightly nodular thickening in the right scrotum with a scurfy condition of the skin over portions of the lesion; on the left, there is a puckered ulcer with moderate thickening of the surrounding skin. These were the lesions as

they appeared 76 days after inoculation. Subsequently the thickening or induration in the skin became diminished, but instead of healing, the lesions spread in an irregular way, producing a peculiar serpiginous necrosis and ulceration over a considerable part of the scrotum (Fig. 44, 118 days after inoculation). There was no suggestion of an acute inflammatory process associated with these changes, and spirochetes were present in fluid drawn from about the lesions.

The next series of photographs (Figs. 45 to 48) shows successive stages in the transformation of a diffuse scrotal infection which reverses somewhat the sequence of events illustrated in the preceding series. The reaction began here as a diffuse infiltration with exfoliation of the epithelial covering of the scrotum (Fig. 45, 53 days after inoculation). After a time, the scaling, or exfoliation, ceased, and the skin became very smooth and translucent. At the same time, the skin became diffusely indurated, and circumscribed nodules appeared in the subcutaneous tissues on both sides. These nodules grew rather slowly, and on the 151st day after inoculation, the condition presented was that shown in Fig. 46. The subcutaneous nodule in the left scrotum was later excised, but the infection in the scrotum persisted in spite of the operation. The nodule on the right continued to grow, and as it developed, the infiltration in the skin diminished. There was, however, a slight recurrence of the exfoliative reaction (Figs. 47 and 48, 165 and 172 days after inoculation).

No attempt can be made to indicate the diversity of conditions to which the specific reaction in the scrotum may lead. By comparing and following the various phases and stages of the reaction, it will be seen that the elements in these reactions are the same in all cases; all show a vascular reaction, a certain amount of exudation, infiltration, and proliferation together with secondary necrosis and ulceration or exfoliation where the necrosis is superficial. The lesions we regard as but an expression of the operation of these factors in the reaction and the balance obtaining in a given case at a given time. As the response to the specific infection varies in different animals or even in the same animal from time to time, so do the lesions, and the possibilities of such variations are almost infinite. The consideration of this phase of the local infection will be taken up in the second part of this paper.

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EXPLANATION OF PLATES.

The first four illustrations are drawings made from the living animal and are based upon photographs taken at the time. The remaining illustrations are reproductions of untouched photographs. All objects are represented at their natural size. The statements of time refer in all cases to the time after inoculation unless otherwise stated.

PLATE 72.

FIGS. 1 to 4. Successive stages of the reaction to infection in the scrotum following inoculation with a virus emulsion.

FIG. 1. 2 weeks. Localized swelling in the scrotum with vascularization of the area. An unusually characteristic reaction.

FIG. 2. 23 days. Early induration with congestion and petechial hemorrhage from the vessels in the center of the lesions.

FIG. 3. 28 days. Anemic necrosis and exfoliation.

FIG. 4. 41 days. Circumscribed and indurated chancres with marked central necrosis and ulceration.

PLATE 73.

FIGS. 5 to 8. Development of scrotal chancres from focal infiltrations following inoculation with a virus emulsion.

FIG. 5. 1 week. Minute focus of induration in the right scrotum and diffuse infiltration with vascularization in the left.

FIG. 6. 19 days. Circumscribed areas of infiltration and induration. The skin over the center is becoming thin and translucent; slight congestion and hemorrhage on the right.

FIG. 7. 28 days. Surface necrosis and exfoliation.

FIG. 8. 43 days. Chancres with well developed collars of induration and marked central necrosis and ulceration.

PLATE 74.

FIGS. 9 to 12. An intense reaction to infection with the development of circumscribed indurated chancres and subsequent extension of the lesions following implantation in the scrotum.

FIG. 9. 10 days. The early syphilitic reaction following inoculation by implantation. There are diffuse edema and congestion of the scrotum and the implants are surrounded by a narrow zone of induration, while the skin over their center has been converted into hard dry crusts.

FIG. 10. 13 days. The early chancre. Note especially the well marked collars of induration and the tendency to exfoliation in the right scrotum.

FIG. 11. 22 days. The diffuse reaction has persisted and the edema has given place to infiltration and thickening of the scrotum.

FIG. 12. 36 days. Large indurated chancres. Diffuse extension of the reaction with widespread necrosis and ulceration of the scrotum.

PLATE 75.

FIGS. 13 to 16. A usual case of scrotal reaction showing cyclic changes in the development of the lesions. Scrotal implantation.

FIG. 13. 14 days. Typical circumscribed lesions with depressed ulcers; edema and congestion of the scrotum.

FIG. 14. 21 days. The lesions have decreased in size; edema and congestion have subsided.

FIG. 15. 28 days. Renewed growth of the chancres and reappearance of scrotal edema.

FIG. 16. 57 days. Large indurated chancres with well marked central necrosis and the formation of ulcers.

PLATE 76.

FIGS. 17 to 20. Deep seated scrotal infection, characterized by slow growth of the lesions and late involvement of the skin. Implantation.

FIG. 17. 34 days. An early stage of the reaction. Skin but slightly involved.

FIG. 18. 41 days. Involvement of the skin with obliteration of papillæ and beginning skin necrosis.

FIG. 19. 44 days. A very active advance of the process indicated in Fig. 18.

FIG. 20. 52 days. The reaction is still quite active. The lesions are extending laterally and the central necrosis is increasing.

PLATE 77.

FIGS. 21 to 26. Multiple foci of primary reaction. Multiple chancres. Implantation.

FIG. 21. 14 days. A well marked accessory focus of reaction at the point of incision of the scrotum on the right.

FIG. 22. 36 days. Overgrowth of the accessory chancre by the main focus of reaction.

FIG. 23. 29 days. Quadruple foci of reaction. The accessory lesion on the right is already regressing.

FIG. 24. 29 days. Triple and double foci of reaction.

FIG. 25. 49 days. Subsequent development of chancres from four of the five initial foci of reaction in Fig. 24.

FIG. 26. 47 days. Quadruple chancres. An unusual case of equal reaction about all of four centers of reaction.

PLATE 78.

FIGS. 27 to 32. Variations and irregularities of the specific reaction. Edema, congestion, and acute inflammatory reactions.

FIG. 27. 16 days. Early circumscribed chancres with diffuse congestion and marked edema of the scrotum. Spirochetes recovered from the point indicated by the arrow on the right.

Fig. 28. 25 days. Focal lesions with acute edema and congestion of the scrotum.

Fig. 29. 39 days. Marked growth of the focal lesions following the exudative reaction in the scrotum.

Fig. 30. 102 days. Sudden development of edema in the scrotum which was the seat of an old indurated lesion. (Compare left.)

Fig. 31. 11 days. An intense granulomatous reaction associated with edema and marked congestion of the scrotum, the exact nature of which is uncertain (see text).

Fig. 32. 50 days. The same animal as in Fig. 31, showing the development of typical indurated chancres following partial regression of the previous lesions.

PLATE 79.

Figs. 33 to 36. Successive stages of an atypical granulomatous reaction.

Fig. 33. 35 days. Simple ulcers of the scrotum with slight induration about their margins and base.

Fig. 34. 83 days. Slow but characteristic development of the lesion in the left scrotum. Regression of the lesion on the right with the formation of a small subcutaneous nodule underneath the original lesion.

Fig. 35. 139 days. Continued growth of the lesion on the left while that on the right has diminished in size.

Fig. 36. 188 days. Complete healing of the original skin lesions with the development of active subcutaneous nodules on both sides.

PLATE 80.

Figs. 37 to 40. Successive stages of an irregular scrotal reaction.

Fig. 37. 36 days. Patches of superficial induration in the skin with necrosis and exfoliation.

Fig. 38. 92 days. The patch of induration on the left is still present, while that on the right has almost completely resolved. Subcutaneous nodules forming on both sides.

Fig. 39. 106 days. On the right, there is a small patch of glassy induration in the skin and beneath this a small indurated nodule surrounded by a mass of diffusely thickened tissue. On the left, the subcutaneous nodule is developing rapidly.

Fig. 40. 127 days. Active development of both nodules with extension to the skin. Note the recurrence of the indurated patch in the right scrotum.

PLATE 81.

Figs. 41 to 44. Diffuse syphilitic processes following scrotal inoculation.

Fig. 41. 33 days. Slight granulomatous reactions about both the implants and the points of incision in the scrotum. The characteristic feature of the reaction is the spreading necrosis and exfoliation over the areas involved.

FIG. 42. 105 days. Diffuse infiltration about the site of inoculation with superficial necrosis and exfoliation. No typical chancre was ever formed in this animal.

FIG. 43. 76 days. A group of irregular and slightly nodular lesions in the right scrotum with surface necrosis and slight exfoliation. On the left, there is a diffuse infiltration of the scrotum with necrosis over the central area.

FIG. 44. 118 days. Same animal. Spreading serpiginous necrosis involving the areas of infiltration.

<center>PLATE 82.</center>

FIGS. 45 to 48. Successive stages in the transformation of diffuse scrotal lesions.

FIG. 45. 53 days. Diffuse infiltration of the scrotum, slight necrosis, and well marked exfoliation of surface epithelium—the initial lesion.

FIG. 46. 151 days. Marked diffuse infiltration of the skin with obliteration of the papillæ; large granulomatous nodules developing in the subcutaneous tissues.

FIG. 47. 165 days. Subcutaneous nodule removed on the left; that on the right still increasing, while the infiltration of the scrotum is slightly diminished.

FIG. 48. 172 days. The operative wound is practically healed; active infection still persists in the skin on the left; the nodule in the subcutaneous tissues is increasing, but the infiltration of the skin has almost disappeared; there is slight desquamation of surface epithelium.

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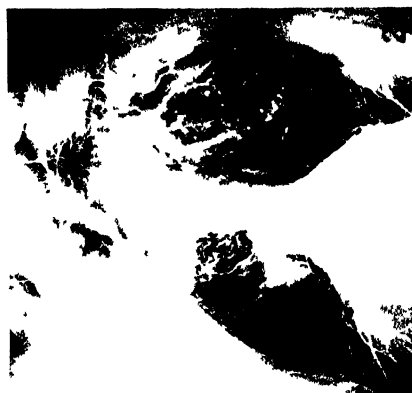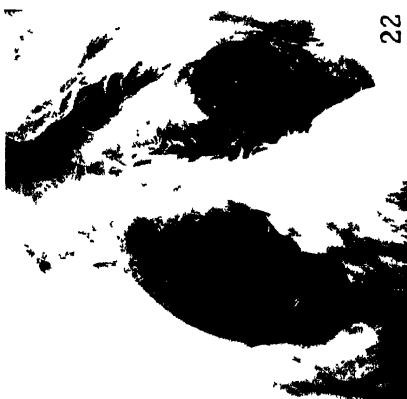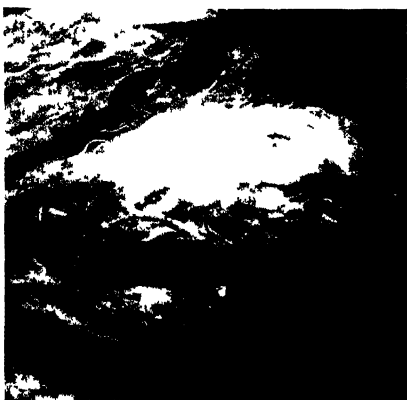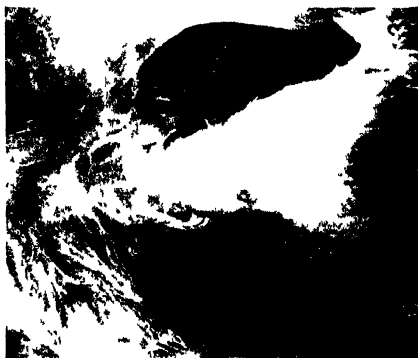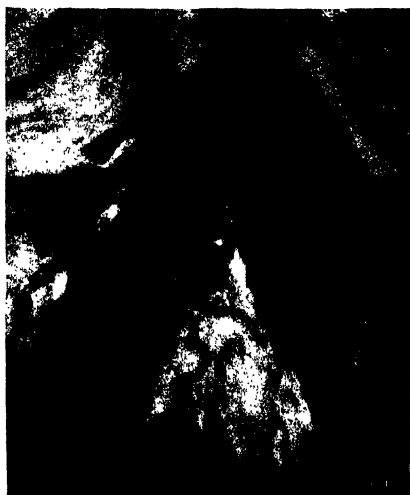
EXPERIMENTAL SYPHILIS IN THE RABBIT.

II. Primary Infection in the Scrotum.

Part 2. Scrotal Lesions and the Character of the Scrotal Infection.

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PLATES 83 TO 89.

In Part 1 of this series, the subject of scrotal syphilis in the rabbit was considered from the standpoint of the local reaction to infection. The various elements in this reaction were noted, and the process was traced from the time of inoculation to the development of characteristic skin lesions. Especial attention was called to the multiform character of these reactions and the influence of various elements in the reaction upon the character of the lesion produced, and while cause and effect were not entirely dissociated, the main emphasis was placed upon the process, and no detailed description of the resulting lesions was attempted. This phase of the subject is, however, of great importance in itself, and a more comprehensive view of the scrotal lesions is essential to a correct understanding and use of the experimental infection.

Primary Lesions of the Scrotum.

A classification of scrotal lesions which would be acceptable from all standpoints is impossible, but in order to simplify the description of these lesions, they may be separated into several general groups based partly upon fundamental differences in growth tendencies and partly upon structural differences in the lesions themselves, neither of which represent sharply defined or entirely fixed characteristics.

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The first division to be made is one which has already been suggested, that of circumscribed and diffuse lesions. The circumscribed chancres include the lesions in which growth takes place from a central focus and tends to be more or less equal in all directions, leading to the development of raised or elevated masses of a circumscribed and indurated character. The diffuse chancres, on the other hand, include lesions which tend to spread laterally and assume a more or less flattened condition. In our experience, chancres of the first group were by far the more common of the two, and since they are more analogous to the classical primary lesions seen in man, they might also be spoken of as typical chancres in contradistinction to the less well defined lesions of a diffuse character.

The variety of primary lesions and the differences which exist between extreme examples of these two groups of lesions are so great that without the intervening links they could hardly be recognized as results of one and the same pathological process. Considerable care has been devoted, therefore, to the mere matter of selection and arrangement of illustrations with a view to giving as accurate an impression of scrotal chancres as possible, both as regards the character of the lesions and the relationship existing between lesions of different types.

Circumscribed or Typical Scrotal Chancres.

The circumscribed, indurated lesions of the scrotum studied by us were lesions which as a class tended to grow to a very large size— much larger than the primary lesions commonly seen in man. These lesions differed according as they arose from one part of the scrotum or another and according to the mode and rate of their development. Based upon these differences, the typical chancres of the scrotum might be divided into two main groups, the nodular and the lenticular or discoid chancres.

Nodular Chancres.—The nodular chancres comprise a large group of lesions whose chief characteristics were an irregular spherical form, a relatively small and sharply circumscribed area of superficial necrosis and ulceration, or the absence of any ulceration, and extreme induration. This general class of chancres may be separated into two

divisions, the ulcerated and the unulcerated chancres, characteristic examples of which are given in Figs. 1 to 6 and 7 to 12.

The nodular chancres appeared to develop from the subpapillary layers of the skin, the subcutaneous tissues, or the outer surface of the tunica vaginalis. They began their existence as more or less spherical masses of induration situated in the deeper portions of the scrotum. The growth of the lesions was comparatively rapid, while necrosis took place more slowly and was of relatively slight extent, at least during the period of active development of the lesion. As the overlying skin became involved, therefore, the area of necrosis was small and a sharply circumscribed depressed ulcer was formed, the margins of which were rounded and intensely indurated (Figs. 1 and 2). These lesions were of a dense fibrous structure, and the small area of central necrosis was usually surrounded by a thick wall of well vascularized living tissue.

The chief variations among the chancres of this class appeared to have their origin in the balance existing between the phenomena of growth and necrosis. The typical condition was that shown in Fig. 1. In a second group of cases (Figs. 2 and 3), growth and necrosis were more nearly balanced and a more extensive destruction of the central portion of the lesion took place. This condition, however, appeared to be referable to a corresponding variation in the structure of the surrounding tissue which in these cases was unusually dense or of almost ivory-like hardness. This condition is suggested in Fig. 2.

The chancres reproduced in Figs. 4 and 5 illustrate another modification commonly seen in chancres of this group occurring either during the period of active growth or as a terminal alteration. The condition was no more than a spreading necrosis which in actively growing lesions produced a unilateral flattening due to cessation of growth in the direction of the skin surface. This alteration was especially noticeable with large and rapidly growing lesions such as those in Figs. 4 and 5. It was a common occurrence, however, with all classes of chancres after active growth had slackened or ceased. In these cases, extension of the necrosis due to a continuation of secondary alterations in the granulomatous tissue resulted in a gradual destruction of the blood vessels and shutting off of the blood supply to the chancre.

This secondary necrosis took place in one of two ways which may be suggested by a comparison of the right and left chancres in Figs. 5 and 6. With large lesions showing a unilateral flattening with surface necrosis, there was a tendency for the spreading necrosis to surround the lesion from the outside (Fig. 5), while in lesions with central necrosis and depressed ulcers, the extension of the necrosis took place radially. This latter type of change, as illustrated in Fig. 6, was, of course, not confined to terminal alterations in chancres but occasionally was seen as an early feature of the lesion and is entirely analogous to the condition shown in Fig. 3.

The second division of nodular chancres differed from the first chiefly in respect to necrosis and ulceration. The two groups of lesions had a common origin and a common structure; in one group, surface necrosis and ulceration of moderate degree were characteristic features of the lesions, while in the other, these secondary alterations were of very limited extent or entirely absent. The photographs reproduced in Figs. 7 to 12 have been arranged to show successive gradations in the tendency to skin involvement and ulceration.

It may be of interest to note that all the chancres used for illustration in Figs. 1 to 12 were produced by a common organism—the Zinsser-Hopkins strain of *Treponema pallidum*. Formerly, lesions of the second type were the more common of the two, while at present the order of frequency is reversed.

Lenticular and Discoid Chancres.—The lenticular and discoid chancres which form the second main division of typical scrotal chancres differed from the first or nodular chancres in being lesions of a more flattened character, in the occurrence of a more widespread necrosis, and in possessing a lesser degree of induration as a rule. Typical examples of lesions of this class are given in Figs. 13 to 24.

The lenticular and discoid chancres appeared to arise mainly from the papillary layers of the skin, and the chief direction of their growth was in a plane parallel with the skin surface. Some of these chancres showed the thicker center and sloping edges of the lenticular lesion, but more commonly the edges were elevated fully as much or even more than the center, giving to the lesions a discoid rather than a lenticular form.

The growth of these chancres was extremely rapid, as a rule, and was associated with widespread skin involvement. Early in the course of their development, the skin over the central portion of the lesion became necrotic, and the extension of this zone of necrosis tended to keep pace with the growth of the lesion, spreading over the surface of the lesion as well as through its deeper parts. In some instances, the necrotic area softened and sloughed away with the formation of a true ulcer, but in others, the necrotic tissue remained firmly attached to the underlying structures, forming a thick imbricated crust (see figures).

Outside the zone of necrosis, the skin covering these chancres was markedly infiltrated and presented an unusually smooth and translucent appearance—a condition which not infrequently extended entirely to the outer edges of the lesions.

These chancres were as a class less indurated than the nodular chancres. In exceptional instances, however, they showed the same dense, fibrous structure and intense induration as nodular chancres (see Fig. 27), but more commonly they were of a mucoid or cellular character, and while these lesions were indurated, their induration was of an elastic quality, giving the impression of tension rather than of hardness.

Rapid growth and widespread necrosis were such striking characteristics of this group of chancres that two sets of illustrations are given to show the extremes to which these processes may go. Figs. 19 to 21 represent chancres produced by inoculation with a virus emulsion. The original lesions in this animal were excised 20 days after inoculation. There was a prompt recurrence, however, and Fig. 19 shows the lesions present 45 days after inoculation, or 25 days after excision of the lesions. From this point on the evolution of these chancres was extremely rapid. Within 20 days, they increased to the size shown in Fig. 20; during the next 3 weeks, growth continued at a somewhat slower rate with a deepening and extension of the necrosis (Fig. 21). The chancre on the left appears here much smaller than that on the right but was in reality almost as large. The appearance is due to foreshortening as only one chancre could be brought squarely in front of the camera.

The other three figures (Figs. 22 to 24) are from chancres produced by implantation. They show an equally rapid growth with the production of lesions which in this case almost lapped the testicles. The necrosis in these chancres was of the type of a dry gangrene spreading diffusely over the surface without a slough and forming heavy crusts composed of concentric layers and rings of ne-

crotic tissue. The figures represent periods of 39, 46, and 60 days respectively after inoculation.

The entire group of lenticular and discoid chancres thus far presented from Figs. 13 to 24 were produced by the Nichols strain of *Treponema pallidum* and show a certain degree of uniformity in type. Some of the more important variations of this class of chancres are indicated in Figs. 25 to 30.

The first two chancres of this group (Figs. 25 and 26) are of a decidedly mucoid character and the skin over the lesions is quite smooth and translucent. One of these chancres is flat while the other shows a tendency towards a more nodular form. In contrast to these lesions which again were products of the Nichols' organism, the two chancres in Figs. 27 and 28 present a decidedly different appearance. They were older lesions but represented a state of development comparable to that of the other two lesions. These chancres were of a dense fibrous character and were intensely indurated. They were produced by the Zinsser-Hopkins strain of *Treponema pallidum*.

The two remaining photographs of this group (Figs. 29 and 30) are given to illustrate chancres of an indolent type or ones which are less vigorous than most of those previously shown. The first of these chancres showed a normal rate of growth but an irregular growth; the lesions were only moderately indurated and there was but slight skin involvement apart from the area of necrosis. The second set of chancres (Fig. 30) showed a very slow and irregular development, with an undermining necrosis. The skin about the lesions was relaxed and there was the merest shell of living and growing tissue. Lesions such as those in Figs. 29 and 30 are more or less constantly at the border-line of regression and their growth may be interrupted by the slightest of causes.

'The chancres thus far described will serve to give a fair impression of the principal chancre types and of the scrotal chancre at its highest state of development. The modifications of these types were so numerous that it would be futile to attempt to describe such a series of lesions. As we pass from these more typical chancres, we come to a group of lesions showing a progressive loss of the characteristics by which we are accustomed to identify primary skin lesions and eventually to lesions which are quite atypical in character. The photographs reproduced in Figs. 31 to 36 are intended to indicate this transition from the typical circumscribed chancres to lesions of a more diffuse and less typical character.

The first three photographs (Figs. 31 to 33) show lesions with all the characteristics of active skin lesions. The chancres in Fig. 31 were virtually thickened plaques with wide shallow ulcers, but the narrow margins as well as the base were markedly indurated. The chancres in Fig. 32 were somewhat less vigorous;

in the right scrotum there was an irregular indurated nodule with a depressed ulcer, and the flattened plaque on the left showed the merest shell of induration. Fig. 33 again shows a nodule or a lenticular thickening in the skin of the right scrotum which fades into the surrounding tissue. On the left, however, there is an extremely small but perfectly characteristic chancre. These photographs represent what was practically the highest point reached in the development of these lesions.

The next three illustrations of this group represent lesions which are decidedly less characteristic. The small ulcer in the right scrotum of the animal shown in Fig. 34 has a definite collar of induration, but on the left, there is little more than a minute sharply defined ulcer with a suggestion of a diffuse thickening in the scrotum. The next lesions (Fig. 35) consisted of irregular areas of thickening with even more irregular areas of ulceration. These lesions represented the height of the local reaction in this animal, and it may be of interest to note that generalized lesions appeared elsewhere just at this time (58 days after inoculation). The final photograph of the series (Fig. 36) shows small nodular lesions in the scrotum of both testicles which persisted for months with almost no change from the condition here shown.

Atypical and Diffuse Lesions of the Scrotum.

The lesions classed as atypical and diffuse include conditions varying from chronic ulcers and nodular thickenings in the scrotum to various inflammatory processes of an ill defined character. As a class, these lesions persist for a long time, and while there is usually no considerable difficulty in demonstrating the specific nature of the lesions, they are subject to frequent transformations which make it very difficult to say whether they should be regarded as primary or as secondary manifestations of infection, or where the line of separation between the two should be placed. At all events, the lesions are the same in either case, and since one group cannot be clearly differentiated from the other, they must be considered here without reference to their primary or secondary character.

Several groups of lesions belonging to this class have already been described and illustrated in Part 1 of this paper. These descriptions of atypical and diffuse scrotal lesions may be supplemented by further examples of lesions of a somewhat different type.

The photographs reproduced in Figs. 37 and 38 represent forms of primary scrotal lesions which were very commonly seen and frequently were the starting point for lesions such as those in Figs. 39 to 42. The first of these photographs

(Fig. 37) was taken 43 days after inoculation and shows a small indurated nodule with an apical ulcer in the right scrotum and an area of diffuse infiltration in the left. The skin in this area showed a loss of papillæ and increased translucency together with the formation of yellowish white scales over its central portion. The second photograph (Fig. 38) shows changes of an analogous character but somewhat more pronounced. The infiltration of the scrotum was more marked on both sides, and on the right there were definite areas of superficial necrosis and exfoliation. This photograph was taken 77 days after inoculation, and just at this time, patches closely resembling those in the right scrotum made their appearance upon the skin at the base of the ears. While neither set of the scrotal lesions shown (Figs. 37 and 38) might conform to the usual conception of a chancre, they were nevertheless the primary lesions of these animals and are just as characteristic of *pallidum* infection in the rabbit as any of the lesions previously described.

Fig. 39 represents another form of scrotal syphilide which occurred either as the starting point of an infection (primary lesion) or as a transformation of other types of lesions. This animal was kept under observation for 29 months after inoculation and never developed lesions more analogous to the ordinary chancre than those shown in Fig. 39, the photograph of which was taken 136 days after inoculation. Altogether, these lesions persisted in essentially the form represented for about 18 months and this animal showed a most marked generalized infection.

The lesions shown in Fig. 40 (190 days after inoculation) are a somewhat different form of the same process as that in Fig. 39. In this animal, there was a diffuse thickening of the scrotum of both testicles, most marked in the dependent portions. On both sides, there was a curved ridge (shown in the photograph only on the right) extending downward and spreading out into a flattened head at the lower end of the scrotum. This ridge and portions of its terminal expansion were profusely covered with scales and thin crusts with erosions here and there.

Finally, there are two illustrations (Figs. 41 and 42) of lesions which in their later transformations tended to revert to a form more like an ordinary chancre. The initial lesions of the animal shown in Fig. 41 were circumscribed nodular lesions with marked congestion and edema of the scrotum. These lesions were of short duration, and by the end of the 5th week after inoculation, they had almost disappeared, leaving a diffuse thickening of the scrotum analogous to that shown in Fig. 37 or 38. 94 days after inoculation (Fig. 41) there was involvement of both testicles (orchitis), diffuse thickening of the scrotum with the formation of fine bran-like scales (shown fairly well on the left), and indolent ulcers on both sides with thickening but no induration about them. These lesions persisted with some further transformations of an equally atypical character up to the time the animal was discarded 11 months after inoculation.

The last photograph of the series (Fig. 42) represents the lesions present in the scrotum of a rabbit 316 days after inoculation. The infection in this animal

began with the formation of lesions almost identical with those shown in Fig. 35 and remained a diffuse infiltrative and exfoliative type of process for upwards of 9 months. It was not until during the 10th month after inoculation that circumscribed lesions of the character shown in Fig. 42 began to appear. On the right, the lesions became confined almost entirely to the nodular mass at the lower end of the scrotum, but at least two-thirds of the left scrotum was the seat of a diffuse infiltration and at times showed a tendency to the formation of scattered exfoliative lesions. (Note the dark colored spots on the scrotum which represent areas of recent exfoliation.) In the midst of this diffuse process, the chancre-like lesion shown in the photograph was formed.

From the standpoint of the human infection, this group of atypical and diffuse lesions of the scrotum is of the utmost importance. They are in many instances lesions which at first glance do not suggest syphilis or at least primary syphilis. They have doubtless been noted by many observers, but very little attention has been paid to them, possibly for the reason that they were interpreted as evidences of a low grade or slight infection which is not necessarily the case.

We have had many rabbits with lesions of this type, a large proportion of which came from the Zinsser-Hopkins strain of *Treponema pallidum*. These animals were of no particular use for therapeutic experiments, and consequently only a comparatively small number of them was kept under observation for any considerable period of time. Those kept were held partly with the hope that they might eventually develop lesions which could be used and partly for the purpose of studying this particular class of infections. It was in this way that we learned what we have about them.

As a class, these atypical and diffuse lesions persisted fully as long or longer than any other class of primary lesions; they contained actively motile spirochetes in abundance, and these organisms were highly virulent which has been demonstrated in two ways, first by the fact that rabbits in which these lesions occurred were frequently the subjects of severe generalized infections, and next that organisms taken from such lesions also produced high grade infections when inoculated into other animals.

General Course of the Local Infection.

Judged upon the basis of the changes which took place in the lesions or by the reaction in the scrotum, the course of the scrotal infection appeared to be fundamentally the same as that in the testicle, modified undoubtedly by differences in the character of the tissue within which or from which the lesions developed. In the scrotum, as in the testicle, there were evidences of periodic changes, but the specific reaction in the scrotum was more stable than that in the testicle and less subject to marked or rapid changes in one direction or another. While one could distinguish between lesions or processes which were diffuse and those which were circumscribed, it was more difficult to draw a sharp line of demarcation between processes which were acute or exudative and those which were proliferative in character.

In general, the initial reaction in the scrotum usually progressed without interruption until a well defined lesion had been produced or for some 2 or 3 weeks at the least. The reaction then assumed an irregular character with periods of growth interrupted by longer or shorter intervals during which little or no change could be detected in the lesions, or during which the lesions appeared to regress. As long as the lesion was merely quiescent or inactive, it retained its appearance unaltered, but when regression set in, the skin about the lesion became relaxed and wrinkled, the induration softened, or the lesion diminished in size. At times, the entire lesion was affected by these changes, while at others, only certain parts of the lesion were affected, or, as in the case of the testicular infection, one portion of the lesion might be actively developing while another was rapidly regressing.

The time element in these changes was most uncertain—some lesions lay dormant over long periods of time (several months) and then grew actively, while others grew steadily for a long time before showing any sign of cessation of activity. Again one group of lesions would develop by more or less regular periods of growth interrupted by short intervals of inaction, while another would show the greatest degree of irregularity. As a rule, a period of growth lasted for a week or more before it was interrupted by an interval of inactivity.

The extent of the change which took place during one of these periods of growth of regression was also a matter of great variation. Growth might be rapid or extremely slow, so rapid that a chancre measuring 2 or more cm. in diameter would develop within as many weeks from the commencement of the reaction, or so slow that no change could be detected from week to week and growth was recognizable only by the change which took place from month to month. Conversely, the phase of regression might be limited to inaction only or might go so far as almost to obliterate the lesion which had been developed and still be followed by an active renewal of growth. However, marked changes in one direction followed by marked changes in the other were extremely uncommon except in cases in which the complete life cycle of the lesion consisted of one such series of changes or was the terminal change in the local infection.

While the extent of the cyclic change was usually limited to a moderate reaction in one direction or the other, the number of such cycles was at times very great, especially in lesions which developed slowly and persisted over long periods of time. As a rule, however, the complete series of such cycles did not exceed three or four and not infrequently was limited to a single cycle.

The duration of the infection in the scrotum as a local or primary focus of infection may be variously stated as from 1 to 18 months. In a limited number of cases the infection developed quickly and subsided with equal rapidity or there was but slight local reaction and this soon subsided. At the other extreme, there were infections which remained firmly established for more than a year, but the average duration of the scrotal chancre was hardly more than 4 to 6 months.

In the majority of instances the infection terminated by degrees. Development gradually ceased and after remaining in a more or less stationary condition for a time, the lesion gradually underwent resolution or healing with the production of a scar, the process consuming on an average from 4 to 6 weeks.

Spirochete Content of Scrotal Chancres.

To demonstrate the presence of spirochetes by the aspiration of fluid from scrotal lesions is usually a very simple operation, but owing to differences in the character and structure of the lesions, comparisons of the numbers of spirochetes in different examples or in different portions of the same lesion are always open to some question. Thus spirochetes are obtained with comparative ease from lesions of a cellular or mucoid character but are more difficult to obtain from fibrous lesions. Again, calculations may be considerably upset by the simple element of dilution which becomes of especial importance where edema exists, and in any case, a negative examination has only a relative significance.

It seems well to emphasize the importance of these points, since in therapeutic experiments, so much stress has been laid upon the number of spirochetes present in lesions at the time of treatment and upon the relative reduction in the number of spirochetes produced by different therapeutic agents. From a wide experience, we realize that no small part of such differences may be traceable to just such factors as those which have been enumerated. This is especially applicable to effects attributed to drugs which produce a marked increase in the fluid content of lesions as a characteristic feature of their action, and, we may add, there are many such drugs, and' these are the ones which, as a rule, produce the most striking apparent reductions in the number of spirochetes present.

With these facts in mind, it may be said that during the early period of chancre growth and as long as a uniform and continuous growth was maintained, actively motile spirochetes could be found in abundance throughout the lesion. Eventually the spirochete content became variable as in other primary lesions. They were numerous and actively motile during periods of active growth or even quiescence, but decreased or even disappeared temporarily from the aspirated fluid during periods of actual regression.

Spirochetes varied likewise with developmental processes or with pathological alterations taking place in different portions of the lesion. As central necrosis developed, they became less numerous towards the center of the lesion or less active and tended to accumu-

late in greatest numbers towards the outer or growing edge. In well developed lesions with necrotic but firm fibrous centers, spirochetes were obtained with difficulty from the central zone and might be obtained only from the outer shell of living tissue. However, in chancres which underwent central softening following necrosis, spirochetes were at times quite numerous in the necrotic debris.

With the progress of the infection and the appearance of irregularly distributed areas of growth and of necrosis, the distribution of spirochetes became likewise irregular; they tended to be numerous and actively motile in regions of most active growth, less numerous and less active in portions of the lesion which were inactive or regressing.

The relation of the spirochete to the size and character of the lesion was less definite. Spirochetes were as abundant and as actively motile in some of the smallest and most insignificant lesions as in the largest and most typical lesion. As a rule, spirochetes were obtained in greater numbers by aspiration of cellular and mucoid lesions or portions of lesions than from fibrous lesions or areas, but, as previously stated, this does not necessarily represent actual differences in the spirochetal content of the respective lesions.

Results Obtained from Scrotal Inoculation.

Before concluding the subject of the scrotal infection in the rabbit, it seems well to refer briefly to the results which one may obtain from scrotal inoculations with *Treponema pallidum* and certain factors which influence these results.

As has already been indicated, there is no particular difficulty in obtaining an infection with organisms which have been thoroughly adapted to the rabbit. With such organisms as we have used, 100 per cent of takes can be obtained with perfect regularity provided one observes a few simple precautions which concern chiefly the state of the virus, the animals used, and the technique of inoculation. As regards the character of the local infection, or more properly the local reaction, uniformity is more difficult to attain, but this also is influenced to a considerable extent by the same factors.

It has been found that the first essential to success is the use of a suitable virus.   As far as mere infection is concerned, material taken from any lesion containing a fair number of actively motile spirochetes will produce infection, but the character of the infection will vary according to the state of the lesion or of the spirochetes in the lesion. Other conditions being equal, the local reaction will take place more promptly and large actively growing chancres will be produced with greater regularity when the material for inoculation is taken from a fresh actively growing lesion, preferably before the height of the first cycle of reaction has been reached.   Material taken from lesions during the ascending phase of the second cycle of an orchitis or of later cycles in the case of skin lesions will sometimes give good results, but at other times, the results are apt to be irregular and less satisfactory as far as the production of chancres is concerned.[1]

In addition, it should be noted that certain strains of *Treponema pallidum* will give results which are more constant and more satisfactory than the results obtained with other organisms.

The second factor in the production of scrotal chancres is the character of the animal used.   The proverbial large rabbit or rabbit with large testicles which is usually interpreted as an old rabbit with a more or less ample or redundant scrotum is a poor subject for the production of scrotal chancres.   The best animals are those just approaching full maturity with well developed but not necessarily large testicles and a thin delicate scrotum.   It is also desirable that the rabbits used be well nourished and free from disease.

Among the various breeds of rabbits, there are great differences in the scrotal reaction to *pallidum* infections.   The Belgian and Flemish giants in particular give poor results, while the small albinos, grays, browns, and Dutch belts on the whole give results of the most satisfactory character.

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[1] It is not to be inferred that the extent of the location reaction in the scrotum is an index of the severity of the infection as a whole, for, as has been pointed out, some of the most severe generalized infections follow the most insignificant local reactions.   Further, there is evidence to the effect that in general a pronounced local reaction in the testicles or scrotum of the rabbit inhibits the development of other focal infections.   This subject will be dealt with in detail in a subsequent paper.

Technique is another factor which plays some part in the success of scrotal inoculations, and the chief element here is simplicity. No antisepsis is necessary, and the use of strong antiseptics is distinctly contraindicated both on account of irritant action upon the scrotum and the possible effect of these substances upon the spirochetes themselves. Many failures in scrotal inoculations have been directly attributable to unnecessary precautions in this direction. Cleanliness, careful operation, and avoidance of undue trauma are the three essentials.

With the use of a suitable virus, good animals, and proper technique, one may attain a high degree of uniformity from scrotal inoculations in as far as the percentage of takes and the production of typical scrotal chancres is concerned. Large indurated chancres were frequently obtained by us in from 75 to 100 per cent of the animals inoculated, but as a rule such lesions were produced in not more than 50 to 75 per cent of the animals. The factor of individuality in the reaction to infection cannot be entirely overcome in *pallidum* inoculations. Just as the typical chancres in a given series of animals will differ from one another, so also one may expect to obtain all degrees of variations in the response of individual animals to a constant set of conditions of inoculation, and irregularities will appear in the results in spite of all that can be done to prevent them.

CONCLUSIONS.

A study of the local infections produced by *Treponema pallidum* in the testicles and in the scrotum of the rabbit leads to the conclusion that the phenomena of infection in the two cases are essentially the same and that such differences as do exist are attributable to differences in the character of the two organs. Upon the basis of these studies, it is possible, therefore, to extend the conclusions which have already been reached in regard to the nature of the local or primary reaction to infection with *Treponema pallidum*.

One is accustomed to think of this reaction as essentially a process of infiltration and proliferation and of the chancre as a circumscribed, indurated, granulomatous lesion. From a consideration of the facts derived from a study of the local infection in both the testicles and the

scrotum, it is apparent, however, that in the rabbit at least, this conception of the syphilitic infection is true only in part and that it is derived more from a study of a condition accomplished than from the process concerned in the production of this condition.

It would seem that there are concerned in the local reaction to infection an element of toxic injury with subsequent degeneration and necrosis which affects especially the lymph and blood vascular systems, a process of exudation and infiltration, and finally proliferation and a mass necrosis due to a progressive destruction of the vessels supplying the affected area. While infiltration, proliferation, and necrosis are the most noticeable features of the local reaction in the usual case of infection, they are, however, no more characteristic than the other processes mentioned and appear to be phenomena of secondary character and importance.

None of these changes bears a fixed relation to the infection, but they are subject to the widest possible variations and in consequence give rise to lesions of the most diverse character in all of which evidences of the same fundamental processes are to be seen. As one feature or another of the reaction becomes more marked, the character of the lesion changes accordingly. Thus the lesion produced in different animals or in the same animal at different periods of the infection may range from lesions in which congestion, edema, and even hemorrhage are the most prominent characteristics to massive granulomatous lesions on the one hand, and from diffuse or ill defined patches of infiltration with desquamation or exfoliation of the surface epithelium to the most sharply circumscribed and intensely indurated nodules on the other.

Finally, it may be said that there is nothing so far as we have been able to discover which clearly differentiates the local reaction at the primary focus of infection from localized reactions to a generalized infection, unless it is the one element of the intensity of the reaction. In many instances, even this distinction is lost, and, as will be brought out in subsequent papers, one reaction is but a repetition of the other, modified to a greater or less extent by the general reaction opposed to the infection and the character of the tissues within which the reaction takes place.

SUMMARY.

From a study of the reaction to scrotal inoculation with *Treponema pallidum* in a large series of rabbits, it was found that the specific reaction presented the following characteristics.

In general, the reaction in the scrotum became apparent within 7 to 14 days after inoculation but was subject to considerable variation. The early reaction took the form of an edematous swelling and congestion associated with a new growth of vessels or of an infiltration with more or less proliferation of fixed tissue cells. These reactions were either confined to a small circumscribed area of the scrotum or were of a diffusely spreading character, and as the infection advanced, the infiltration and proliferation together with such secondary changes as exfoliation, necrosis, and ulceration became the most conspicuous features of the reaction.

The course of the reaction in the scrotum was essentially the same as that in the testicle; that is, it was periodic in character and was marked by a phase of active progression followed by quiescence or regression and renewed activity.

The scrotal reaction resembled that in the testicle also in the varying character of the reaction, appearing at times as a circumscribed focus of reaction and later becoming diffuse, or first as a diffuse reaction which subsequently became more localized.

The lesions produced in consequence of this reaction were of two general types—one a circumscribed indurated granulomatous lesion closely resembling the human chancre, the other a diffuse infiltration more analogous to the secondary skin lesions of man. Both groups of lesions presented the greatest degree of individual variations and possessed no fixed status but were subject to frequent and marked transformations. After a period of from a few weeks to many months, the lesions in the scrotum disappeared spontaneously.

EXPLANATION OF PLATES.

All the illustrations are reproductions of untouched photographs which represent the objects at their natural size. The statements of time given are estimated from the date of inoculation except where otherwise stated.

PLATE 83.

FIGS. 1 to 6. Typical nodular chancres of the scrotum.

FIG. 1. 49 days. Characteristic multinodular chancres with small depressed ulcers.

FIG. 2. 41 days. Intensely indurated chancres with marked central necrosis. Edema and congestion of the scrotum with focal hemorrhages on the left.

FIG. 3. 44 days. Nodular chancres with unusually marked central necrosis and only a comparatively thin shell of living tissue. These features were developmental characteristics.

FIG. 4. 44 days. Large nodular chancres with unilateral flattening. Growing surfaces slightly mucoid in character. Edema of the scrotum.

FIG. 5. 44 days. Large nodular chancres with marked surface flattening of the right chancre due to spreading necrosis.

FIG. 6. 70 days. A vigorous nodular chancre on the left with marked central necrosis of the chancre on the right—a phenomenon of decadence.

PLATE 84.

FIGS. 7 to 12. Nodular chancres of a more spherical type with a lessened tendency to necrosis and ulceration.

FIG. 7. 43 days. Spherical chancres. Spreading surface necrosis on the right, slight necrosis on the left.

FIG. 8. 46 days. Spherical chancres ulcerated and unulcerated.

FIG. 9. 60 days. Spherical chancres. The skin surface on the left still uninvolved.

FIG. 10. 90 days. Irregular nodular chancres with small areas of skin necrosis.

FIG. 11. 89 days. Spherical chancres in the subcutaneous tissues of the scrotum, with no sign of surface necrosis. Diffuse infiltration of the lower end of the scrotum on the left.

FIG. 12. 112 days. Spherical chancres in the deeper layers of the skin with infiltration of the papillary layers but no necrosis.

PLATE 85.

FIGS. 13 to 18. Typical lenticular and discoid chancres of the scrotum.

FIG. 13. 28 days. Rapidly growing lenticular chancres with spreading necrosis of the skin surface. Thick acuminate crusts.

FIG. 14. 28 days. Discoid chancres with deep necrosis and ulceration on the right.

FIG. 15. 38 days. Discoid chancres with deep ulceration and thick, dry crusts. Marked edema of the scrotum.

FIG. 16. 45 days. Typical mucoid chancre with extensive ulceration and infiltration of the skin.

FIG. 17. 39 days. Discoid chancre of more fibrous type.

FIG. 18. 46 days. Discoid chancres with spreading necrosis and thick imbricated crusts.

PLATE 86.

FIGS. 19 to 21. Growth and necrosis of mucoid chancre.

FIG. 19. 45 days.

FIG. 20. 65 days.

FIG. 21. 86 days.

FIGS. 22 to 24. Evolution of a more spherical mucoid chancre.

FIG. 22. 39 days.

FIG. 23. 46 days. Necrosis beginning to spread.

FIG. 24. 60 days. Spreading necrosis with thick imbricated crusts.

PLATE 87.

FIGS. 25 to 30. Modified types of lenticular and discoid chancres.

FIG. 25. 35 days. Lenticular mucoid chancres.

FIG. 26. 40 days. Mucoid chancres tending towards a spherical form.

FIG. 27. 61 days. Lenticular chancres of dense fibrous structure.

FIG. 28. 65 days. Lenticular chancres of dense fibrous structure showing marked necrosis and thick adherent crusts.

FIG. 29. 37 days. Mucoid chancres with moderate induration, slight skin involvement, and undermining necrosis (right).

FIG. 30. 97 days. Old fibrous chancres with undermining necrosis and relaxed skin covering. Inactive.

PLATE 88.

FIGS. 31 to 36. Transitional types of chancre reactions.

FIG. 31. 84 days. Right, a flattened plaque of induration with ulceration extending practically to its edges. Left, an irregular area of induration, necrosis, and ulceration. Both chancres still quite active.

FIG. 32. 73 days. An irregular ulcerated nodule on the right and a small flattened chancre on the left.

FIG. 33. 55 days. Irregular mass of induration on the right and a very small but typical lenticular chancre on the left.

FIG. 34. 36 days. Small lenticular chancre on the right and a small patch of necrosis in the skin on the left.

FIG. 35. 58 days. Irregular patches of necrosis and ulceration with slight infiltration of the surrounding tissues.

FIG. 36. 29 days. Small nodules of induration.

PLATE 89.

FIGS. 37 to 42. Atypical scrotal lesions (chancres (?)).

FIG. 37. 43 days. Right, a small indurated papule with central ulcer. Left, an area of diffuse infiltration and slight desquamation of epithelium.

FIG. 38. 77 days. Diffuse infiltration with superficial necrosis and exfoliation.

FIG. 39. 136 days. Marked infiltration of certain areas of the scrotum with focalized areas of necrosis and ulceration and extensive exfoliation.

FIG. 40. 190 days. Diffuse infiltration of the scrotum with marked exfoliation in certain areas.

FIG. 41. 94 days. Diffuse infiltration of the scrotum, secondary ulceration with slight'induration, and double orchitis.

FIG. 42. 316 days. A late reversion of a diffuse scrotal syphilis to lesions of a circumscribed type.
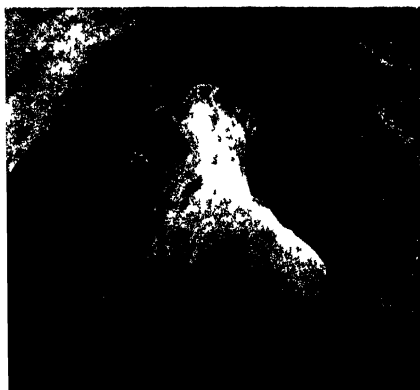
(Brown and Pearce Experimental syph i s in the rabbit   II)
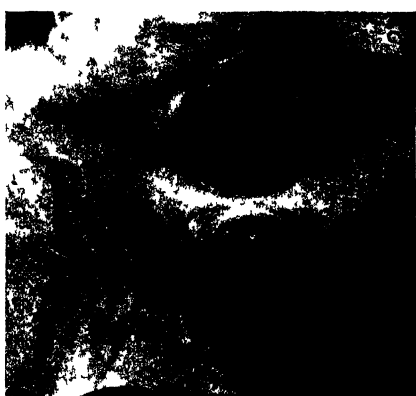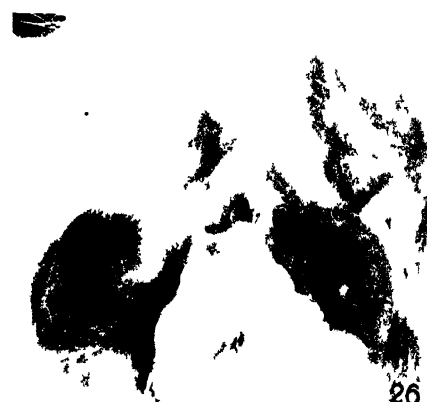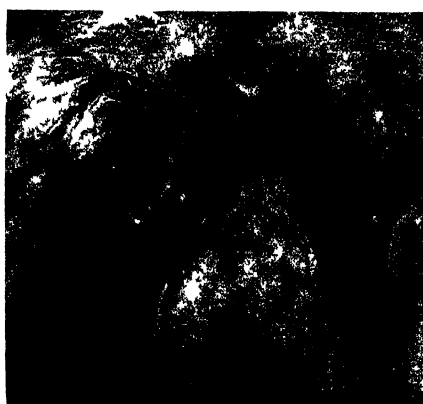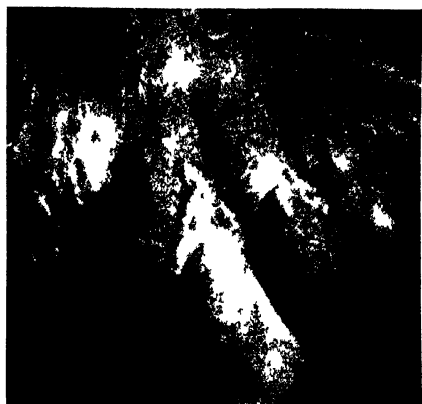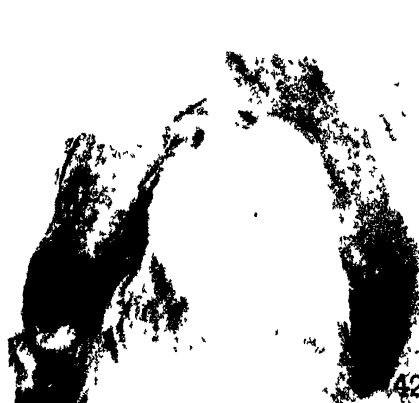
EXPERIMENTAL SYPHILIS IN THE RABBIT.

III. LOCAL DISSEMINATION, LOCAL RECURRENCE, AND INVOLVEMENT OF REGIONAL LYMPHATICS.

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PLATES 90 TO 97.

In preceding papers of this series, the testicular and scrotal infections of *Treponema pallidum* were described from the view-point of a progressive local infection, and in following out this mode of presentation, a number of lesions were described as transformations or extensions of the local infection without any attempt to differentiate such lesions from lesions of a similar character which might arise as a result of local dissemination of the infection rather than from direct extension or transformation of primary lesions. It was pointed out, however, that there were many lesions of the testicles and scrotum which occupied a position of uncertainty with reference to the primary infection and that it could not be determined in all cases whether the lesions present represented actual transformations and extensions of a primary infection or had arisen as a result of local dissemination.

In brief, it was recognized that there were two groups of lesions in the testicles and scrotum which could not be clearly separated from one another. One group is composed of modified and persistent forms of primary lesions together with direct extensions and true recurrences, while the other represents lesions which owe their origin to local or regional dissemination of the virus.

These lesions are of especial interest in that they mark a transition between conditions which can be clearly recognized as primary manifestations of infection and conditions which represent true generalized infection. The subjects of chief interest in this connection are those of local spread and local dissemination of the infection, recurrent and

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consecutive lesions, and involvement of regional lymphatics. References to this phase of experimental syphilis have been very few and for the most part rather vague.

Attention was called to the phenomenon of local metastasis by Uhlenhuth and Mulzer and by Truffi at about the same time. Truffi (1) reported a case of a granulomatous nodule arising in the epididymis and another of a lesion in the tunics following primary infections of the scrotum. Uhlenhuth and Mulzer (2) reported metastatic infection from one testicle to the other testicle and scrotum and a second case of metastasis to an uninoculated testicle after removal of the inoculated testicle. These observations were made in 1909 and 1910, and since that time, metastases of this kind have frequently been cited as evidence of generalization of the infection in the experimental animal.

Involvement of the inguinal lymph nodes in infected animals has likewise been noted both as an analogy to the human infection and as proof of generalization. This condition was first reported by Ossola (3) (1909) who was able to demonstrate spirochetes in sections of the inguinal nodes of animals inoculated in the scrotum. These findings were confirmed by Truffi (4) and the virulence of the organisms was proven by animal inoculation.

Other than observations of this kind, very little has been recorded concerning this border-line group of syphilitic manifestations.

Local or Regional Dissemination.

In animals infected with *Treponema pallidum*, one must recognize the existence of two opposing influences, the tendency of the organism to spread beyond the local confines of the point of inoculation and the opposing reaction on the part of the animal. Some extension of the infection and of the zone of local reaction occurs in all animals even with the most sharply circumscribed lesions, but the extent of the two processes does not run parallel.

The details of the local changes are either masked in a continuous and widespread reaction or else there are no lesions to mark the extension of the infection beyond the zone of primary reaction. In exceptional instances, however, cases do arise which when linked together furnish some interesting data concerning the spread, or dissemination, of the infection within the regions adjacent to the point of inoculation.

In addition to the widespread reaction commonly observed in the course of development of primary lesions, there were three general

types of local reaction which we were able to make out in connection with the dissemination of the infection in the testicles and scrotum: first, a diffuse reaction with the formation of multiple foci of secondary reaction arising subsequent to the development of the primary lesion; second, a reaction characterized by the formation of lesions having a perivascular arrangement; and third, dissemination of the infection with the production of multiple lesions showing no demonstrable connection with the primary focus of infection. While lesions of these three classes occurred in both the scrotum and testicles, they were most clearly definable in the scrotum, and for that reason, the discussion of the subject will be confined to the scrotal infection.

Diffuse Secondary Reaction with the Formation of Multiple Focal Lesions.—One of the most common forms of local dissemination observed was one which took place with a more or less diffuse reaction about a primary focus of infection and led to the formation of multiple secondary lesions which tended to become fused into a single chancre-like mass. This form of reaction is apt to be confusing and it is only in cases where the successive steps in the process can be followed that the ultimate lesion can be distinguished in any way from a true primary lesion. A typical case of this kind is illustrated in Figs. 1 to 3.

The primary lesions in this animal were small circumscribed chancres of a rather indolent character and for approximately 2 months there was nothing unusual about the local infection. A slight diffuse thickening of the scrotum then developed about these lesions and a number of small thickened plaques were formed here and there in the scrotum. These plaques developed into nodules and the diffuse reaction in the scrotum increased after the manner shown in Fig. 1. At the time this photograph was taken, 85 days after inoculation, the primary lesions themselves (indicated by arrows) had begun to grow actively; the entire scrotum was markedly thickened and slightly reddened, and the focal lesions showed signs of necrosis and ulceration. The development of these lesions continued as indicated by Figs. 2 (91 days after inoculation) and 3 (113 days after inoculation) with the formation of an increasing number of secondary foci which tended to fuse into a single large granulomatous lesion indistinguishable in character from an ordinary chancre.

It should be noted here that the development of this series of lesions in the scrotum, occurring 2 months after inoculation, coincided with the appearance of similar granulomatous lesions on the

feet and legs of this animal and the two sets of lesions ran a parallel course of development.

This is but one of many cases of the kind which have come under our observation but will serve to indicate the possibilities for confusion of primary lesions and lesions which owe their origin to dissemination from a primary focus of infection.

Local Dissemination with the Formation of Perivascular Lesions.— The second form of local dissemination to be considered differed from the first chiefly in that the development of the lesions was less obscured and the lesions themselves were of a very distinctive type.

In taking up this group of lesions, we may refer first to a form of direct extension from the primary lesions which was observed in cases in which the initial lesion was slow in developing or of slight extent and spread of the infection along the perivascular tissues could be detected even before the reaction at the site of inoculation had become well established. The animal shown in Fig. 4 was one of a group inoculated with a virus known to contain large numbers of spirochetes of low vitality. Following inoculation, the implant became soft and cheesy in character and no specific reaction could be detected for about 6 weeks. Finally, a narrow line of induration developed at the outer margin of the implant in the right scrotum and fine thread-like lines of infiltration immediately began to spread along the course of the blood vessels leading away from this area as shown in the photograph (Fig. 4) taken 56 days after inoculation. These lines of infiltration grew until they formed a series of cords about 1 mm. in diameter with node-like thickenings at various points. Later, the intervening sections in the distal portions of the cords disappeared, leaving a series of isolated shotty nodules, while the parts nearest the implant became fused into a single irregular granulomatous mass.

In the left scrotum, a similar type of reaction took place except that the lines of perivascular extension were not so clearly defined. When first discovered, there were numerous isolated points of induration diffusely scattered through the scrotum, many of them occupying a perivascular position. Soon after the appearance of these disseminated lesions in the scrotum, lesions developed upon the sheath and about the anus of this animal.

We have in this animal what appears to be a twofold process, first a gradual but direct extension of the primary reaction along certain definite lines, and second a widespread development of focal lesions resulting from dissemination of spirochetes with which the development of the primary lesion had not kept pace.

A somewhat clearer example of local dissemination with the production of peri-vascular lesions is to be found in the animal shown in Figs. 5 and 6 which belonged to the same series as that shown in Figs. 1 to 3. The initial lesions were again small ulcerated chancres with slight induration. These lesions were soon trans-formed into a moderate diffuse thickening of the ventral surface of the scrotum, while the ulcers healed, leaving small puckered scars (Fig. 5). About 6 weeks after inoculation, a series of small shotty nodules was palpable in the dorsal fold of the scrotum. These nodules were just visible as translucent points distributed in chains along the course of the blood vessels extending centrally from the thick-ened patches in the scrotum. They gradually increased, and at the time the first photograph was taken (Fig. 5, 63 days after inoculation), there were numer-ous nodules distributed all through the scrotum, including the area beneath the scar, and the course of the blood vessels in the dorsal fold was marked by a series of indurated lines and nodes (Fig. 5). While these lesions are shown on only one side, the condition on the other was exactly the same.

During the next 3 weeks, the character of the lesions changed materially. Many of the papular lesions disappeared, leaving only a few perivascular lesions in the dorsal portion of the scrotum (Fig. 6). The chief seat of the specific reac-tion shifted back to the ventral surface of the scrotum which showed a heavy shell of doughy thickening with a few large masses of induration. The subse-quent changes were much the same as those shown in the first series of photo-graphs (Figs. 1 to 3).

Again we may note that the occurrence of these papular lesions in the scrotum coincided with the development of a periosteal lesion at the distal end of the left ulna.

While lesions of the type described were observed in all parts of the scrotum, the seat of predilection was the lower end of the dorsal fold, and the occurrence of one or more lesions in this location was quite common. When the lesions were multiple or in the form of cords, they frequently followed a definite line of distribution or exten-sion from the point of inoculation or from the primary lesion towards the body axis.

Local Dissemination with the Formation of Multiple Secondary Lesions.—The third group of disseminated lesions to be described differed from the first group only in that they were clearly focalized lesions from the beginning and from those of the second group in that they showed no apparent perivascular relation and no definite con-nection with the parent lesion. These lesions usually appeared as multiple papules or plaques and tended to develop into multiple lesions of a chancre-like character. Sometimes there were only one

or two such lesions, but at others, there were as many as six or eight. The animal shown in Fig. 7 had six distinct lesions of different sizes and stages of development in each scrotum, among which it would be rather difficult to identify the primary lesion.   As in the previous cases, they tended ultimately to diminish in number, and the reaction usually became centered in one or two lesions which developed to a considerable size, overgrowing and fusing with the surrounding lesions.

It is probable that lesions of this class have in general been regarded as multiple primary lesions, but as a rule they do not begin to appear until after a primary lesion of some sort has developed at the immediate point of inoculation or for some 6 weeks or more after inoculation, and while they may become the main lesions present, they are not primary in the sense of lesions developing as a result of simultaneous localization of the infection.   This distinction may or may not be of importance according to the significance which may be attached to primary and so called secondary lesions.

Recurrent Lesions.

The lesions which develop in the testicles and scrotum of an infected animal subsequent to the regression or healing of an initial lesion have many points of interest, but their chief importance lies in their bearing upon problems of local dissemination and the persistence of active infection when once the local lesions have completely disappeared.   In its application to experimental therapy, there are few subjects of greater importance than this, and while we cannot enter into this phase of the subject with the thoroughness which it demands, the therapeutic importance of recurrent lesions may be kept in mind.

The lesions which develop after spontaneous healing of an initial lesion and those which appear after healing which has been induced by artificial means are identical in all respects.   Induced healing, as by the use of therapeutic agents, offers the advantage, however, of clearer definition between the process of healing and recurrence, and for this reason we shall use cases for illustration in which the healing of the original lesion was experimentally induced.

As an introduction to the subject of true recurrence, it may be pointed out that the power of recovery in an early and actively developing scrotal chancre is very great and that regression may be carried almost to the point of complete healing and be followed by prompt regeneration of the lesion. This fact is illustrated in Figs. 8 to 10. Fig. 8 shows two chancres 40 days after inoculation at which time the animal was treated. The lesions regressed rapidly, and 2 weeks later, there was an irregular thickened mass in the right scrotum and only a small ulcer with no induration in the left (Fig. 9). At this point, regeneration set in, and in 2 weeks more, chancres showing the same characteristics as the original lesions were produced (Fig. 10). This is the result usually obtained when regression stopped short of complete healing.

Once complete healing of a lesion was accomplished, it was rare that another lesion developed from exactly the same point. The four most common locations for recurrent lesions were the tissues beneath the scar, the edges of the scar, the dorsal folds of the scrotum, and the testicles. The lesions developing in the scar were usually no more than indurated plaques, although typical chancres were observed which formed upon the basis of an old lesion.

In the tissues beneath the scar, the recurrent lesions usually took the form of indurated nodules, some of which might develop to considerable size and involve the overlying skin with the formation of chancre-like lesions.

A typical example of this form of recurrence is given in Figs. 11 to 13. Fig. 11 is the original chancre 29 days after inoculation, Fig. 12 the healed lesions, and Fig. 13 the multinodular recurrence with an ulcerated chancre-like lesion in the left scrotum 78 days after treatment.

A more common form of deep scrotal recurrence is that shown in Figs. 14 to 16. Recurrence in this animal did not take place until 55 days after treatment, and the lesions formed were small indurated nodules which were at first confined to the subcutaneous tissues. Later, the skin became involved, and a diffuse induration with multiple focal lesions extended over a large portion of the scrotum of both testicles (Fig. 16). During 4 months observation, no typical chancre-like lesion developed.

Another form of recurrence which was frequently seen in the scrotum is that shown in Figs. 17 to 19. This animal was treated 37 days after inoculation and the chancres healed promptly. 55 days after treatment, there were a few small indurated nodules at the outer edges of the scars on both sides. The scars themselves were smooth and thin (Fig. 19, 77 days after treatment). Subsequently other lesions of the same character appeared elsewhere in the scrotum, but they remained for only a short time and none of them developed to a size much larger than that shown in the photograph.

In some instances these recurrent lesions even though they were far removed from the seat of the original chancre showed a form of development identical with that of a chancre. Such a case is illustrated in Figs. 20 to 22. Recurrence took place in this animal 127 days after treatment, and the lesions appeared as single, minute, indurated nodules situated at the lower end of the dorsal fold of the scrotum; they were bilateral and symmetrical. These lesions grew rapidly and assumed the appearance of typical chancres (Fig. 22, 133 days after treatment).

In addition to the various forms of nodular lesions described, diffuse lesions of various types were observed in many animals. A case of this type which combines something of the characteristics of a diffuse and a focal process is shown in Figs. 23 to 25. This animal was treated 48 days after inoculation (Fig. 23). The lesions regressed very rapidly until they had almost healed, when a diffuse infiltration appeared over a large part of the scrotum (Fig. 24). Healing of the initial lesion continued, however, with the formation of an ordinary looking scar, and the thickening of the scrotum diminished (compare Figs. 24 and 25). 28 days after treatment, a series of slightly reddened and translucent points appeared around the margins of the scars (Fig. 25, 35 days after treatment). These spread rapidly, producing a slight diffuse induration in the scrotum with scaly points here and there.

A final form of recurrence to be noted is that which takes place in the testicle following the healing of a scrotal lesion, and it may be mentioned in this connection that such recurrences were quite common as were recurrences of testicular lesions in the scrotum. The most common locations of recurrent lesions in the testicle were the head of the epididymis, the tail of the epididymis, the mediastinum testis, and the tunics, or the same positions in which primary testicular infections tend to become localized.

This form of recurrence is illustrated in Figs. 26 to 28. The chancres in the scrotum of this animal healed completely in 3 weeks. 1 week later, there was a definite swelling of both testicles and indurated nodules were found in the head of the epididymis on both sides (Fig. 28); the scrotum remained normal.

The time of occurrence and the relative frequence of the various types of recurrent lesions will not be entered into. It seems well to mention, however, that the more characteristic lesions usually appeared early after the healing of the original lesion, while the smaller, less easily recognizable ones were more delayed in their development. The time of recurrence in our series of animals varied anywhere from a few days to many months, and so many circumstances enter into

estimations of the possibilities of local or regional recurrence that definite statements as to time limits cannot be made upon the basis of the data at present available.

To emphasize the difficulties attendant upon observations as to recurrence of local infections, the case illustrated in Figs. 29 to 31 may be cited. Following treatment, the chancres in this animal (Fig. 29) healed rather slowly, and healing was not complete at the end of 6 weeks (Fig. 30); however, smooth, thin scars were eventually formed. 63 days after treatment, two small areas of thickening appeared at the edge of the scar in the right scrotum but disappeared within a few days and infection could not be proven. 105 days after treatment, the extremely small nodule shown in Fig. 31 appeared in the left scrotum and proved to be syphilitic. There was a slight local edema associated with this nodule, but the nodule did not increase in size and persisted for only a short time. No other local lesions were found, but the animal developed a specific iritis 192 days after treatment.

A second animal of a similar character which came under our observation was one in which minute points of induration appeared in the dorsal folds of the scrotum 107 days after treatment. These nodules persisted for 8 months with very little change and no other lesions appeared for some time, but eventually a generalized rash developed upon the face and ears.

It will be noted that the lesions described as recurrences are not unlike some of the conditions previously described in connection with transformations of primary lesion and especially like those resulting from local dissemination. In fact, transformation, dissemination, and recurrence are all overlapping processes with no sharp line of separation between them, and each of these phenomena throws some light upon the others.

Involvement of Regional Lymphatics.

Enlargement and induration of the inguinal lymph nodes has been noted by several observers in connection with *pallidum* infections of both the testicles and the scrotum of the rabbit, and organisms have been demonstrated in these nodes in some instances. There is, however, little definite information concerning the relation of lymphadenitis to the experimental infection. The subject is a very broad one in itself and will not be discussed in detail at the present time. This aspect of the problem of experimental syphilis will be taken up in a separate paper, and we shall confine ourselves here to

general statements concerning involvement of the inguinal nodes in primary infections of the testicle and scrotum as a phase of local dissemination. ·

In normal rabbits, the inguinal glands are small, ovoid, or flattened masses which are barely visible or palpable (Fig. 32). From examination of a large number of rabbits, it was found that enlargement and induration of these nodes was inconstant in testicular infections and depended largely upon the extent of the local infection. In cases in which localized infection was confined to the testicles proper, there was little or no involvement of the inguinal glands, but when the infection extended to the scrotum, these glands became involved just as in cases of primary scrotal infection.

Character of the Involvement.—Localized infection in the scrotum was found to be invariably associated with marked inguinal lymphadenitis. Following inoculation or extension of an infection to the scrotum, pathological changes in the inguinal nodes could be detected almost immediately. The change began as an acute swelling, part of which might have been due to a non-syphilitic reaction but was merged indistinguishably with the syphilitic reaction which progressed rapidly until the affected glands were several times their normal size. During the early stages of the enlargement, the glands remained rather soft but gradually became harder and finally reached a condition of extreme induration. In some instances, the stage of acute swelling was absent; in these cases, the glands first became hard and shotty and then underwent a gradual enlargement.

The exact change which occurred in individual animals was found to be very variable, but a general idea of the inguinal adenopathy may be gained from an examination of the photographs reproduced in Figs. 32 to 43 which represent various forms and degrees of involvement.

As may be seen by reference to Figs. 33 and 34, a well developed adenitis was present in cases of active infection within a few days after inoculation. The 5 day example given in Fig. 33 is a rather pronounced instance of acute bilateral swelling more marked on the right than on the left. In Fig. 34 (7 days after inoculation), the change is not so apparent, especially on the right. Upon close examination, however, it will be seen that there are three nodes in this region, the largest of which is situated immediately over the cord and hence does not stand out so prominently as the one on the left.

The phase of acute swelling as exemplified in these two illustrations frequently reached its height by the end of the 1st or 2nd week after inoculation. The induration, however, continued to increase for some time and was not infrequently associated with a slight temporary decrease in the size of the gland. When fully developed, the inguinal adenopathy presented a variety of appearances. The glands chiefly affected were those immediately adjacent to the inguinal ring. As a rule, the involvement was well marked and of the same character on the two sides but rarely equal (see figures); occasionally the nodes of one side were much more enlarged and indurated than those of the other (Fig. 36). In some instances, the nodes affected were situated higher in the groin (Fig. 37), and even those of the flank might be enlarged and indurated.

Usually there were only one or two nodes involved on each side, and these were of a rounded or oval shape. Sometimes a number of small nodes were grouped together in one mass (Fig. 40); occasionally the nodes were flattened or lay in positions which made it difficult to appreciate the full extent of their enlargement. This may be seen by a comparison of the left inguinals in Figs. 41 and 42, in the latter of which the node has been picked up in order to show its actual size. This photograph also serves to convey some idea of the degree of induration in these nodes which when fully developed were extremely hard and of a semitranslucent appearance.

Significance of Lymphoid Involvement.—As will be noted by a comparison of the various figures, there was no apparent relation between the size of the scrotal lesion and the extent of the lymphadenitis. It frequently happened that the relative involvement of scrotum and nodes was in inverse proportion. The force of this statement is well brought out by Fig. 43 which shows a most pronounced lymphadenitis in an animal with comparatively slight scrotal lesions. Involvement of the inguinal glands appeared to be more an index of the activity of the scrotal infection and of the spread of the infection than of the extent of the local reaction in the scrotum.[1]

[1] In addition to the observations here recorded, this statement is based upon a long series of experiments in which the presence or absence of localized infection in the lymph nodes has been determined at periods of from a few hours after inoculation to more than 2½ years. The details of this work will be reported later. It is sufficient at present to say that enlargement and induration of lymph nodes in the rabbit always signify existent infection of the nodes.

As long as there was active infection in the scrotum, the inguinal nodes were apt to remain enlarged and indurated, but as the scrotal infection subsided, the nodes usually softened and diminished in size. Occasionally these changes occurred even before any very definite change in the scrotal lesions was noted, while in other instances, the adenopathy persisted even though the scrotal lesions showed decided evidence of regression or after they had healed completely.

Enlargement and induration of the inguinal lymph nodes in the rabbit were so constantly associated with scrotal infection that they could be used as diagnostic signs when any doubt existed as to the presence or absence of specific infection in the scrotum; not only this, but these signs could be so used before a scrotal lesion had had an opportunity to develop. The chief interest in involvement of the regional lymphatics, however, is their significance in connection with dissemination or generalization of the infection.

SUMMARY AND CONCLUSIONS.

From a study of the phenomena of the primary infection on the one hand, and the phenomena of local spread, or dissemination, on the other, it is seen that a multiplicity of lesions develops in the testicle and scrotum of the rabbit which have much the same characteristics irrespective of their origin. Some of these lesions are clearly recognizable as primary lesions or parts of a primary reaction to infection, while others are just as clearly the results of dissemination of the virus from a primary focus of infection or correspond with lesions which are commonly spoken of as secondary lesions. The effort to draw a sharp line of distinction between these two groups of lesions or between a primary and a secondary stage of infection in the rabbit, however, would be largely an arbitrary procedure. The fact is that the tissues of the scrotum and testicle of the rabbit constitute favorable surroundings for the localization and development of *pallidum* infections. Under ordinary circumstances, a large part of the reaction to infection which expresses itself in the formation of lesions recognizable by ordinary methods of examination takes place in these tissues. These lesions present certain broad and general characteristics without regard to whether they are primary or secondary in origin; the reaction is merely a reaction to a syphilitic infection which in either case may assume the most diverse character.

Further, it would appear that in rabbits infected with such strains of *Treponema pallidum* as we have used, the virus is never confined to the area occupied by the so called primary lesion, or chancre, but always spreads and always gives rise to a regional adenopathy. There may be no lesions to indicate the progress of this dissemination, but an examination of the inguinal nodes shows that dissemination occurs very soon after inoculation, and a *pallidum* reaction may be detected in these glands even before infection can be recognized in the scrotum. Subsequently lesions develop in all parts of the scrotum and testicle, sometimes involving the entire testicle or scrotum, and at others, forming focalized lesions with an especial predilection for certain locations such as the epididymis, the mediastinum testis, the tunics, and the dorsal folds of the scrotum. In some instances, more or less continuous lesions form along the course of the perivascular lymphatics, suggesting that this is one path taken in the dissemination of the organism. It is probable, however, that lesions of a gross character develop more as a result of accumulation of spirochetes than of mere invasion of the lymphatics since they are not a constant accompaniment of the local infection, while invasion of the lymphatics and extension of the infection to the regional lymph nodes occur in all cases.

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EXPLANATION OF PLATES.

The illustrations are all reproductions of untouched photographs which represent the objects at their natural size. Except where otherwise indicated, the statements of time are all estimated from the date of inoculation.

PLATE 90.

Figs. 1 to 6. Lesions resulting from local extension, or local dissemination, in the scrotum.

Fig. 1. 85 days. Diffuse involvement of the scrotum with the formation of multiple focal lesions. The primary lesions are marked by arrows.

Fig. 2. Same animal. 91 days. Subsidence of the diffuse reaction with rapid evolution of the focal lesions.

Fig. 3. Same animal. 113 days. Final stage in the transformation of the disseminated lesions into a chancre-like lesion.

Fig. 4. 56 days. Perivascular lesions extending from the outer edge of an implant about which there has been very slight reaction. The implant is marked by an arrow.

Fig. 5. 63 days. A diffuse thickening of the scrotum with perivascular infiltrations along the vessels of the dorsal fold. The seat of the original chancres is indicated by the tiny crust on the right and the marked depression on the left.

Fig. 6. Same animal. 85 days. A later stage of the same lesions.

PLATE 91.

Fig. 7. 69 days. Multiple focal lesions of the scrotum with tendency to chancre-like transformations.

PLATE 92.

Figs. 8 to 10. Recovery of incompletely healed chancres after treatment.

Fig. 8. 40 days. The chancres at the time of treatment.

Fig. 9. 2 weeks after treatment, marking the extent of the regression produced.

Fig. 10. 4 weeks after treatment. Compare the regeneration effected with the original lesions and with the extent of the regression produced in the two chancres.

Figs. 11 to 13. Local recurrence after complete healing of scrotal chancres.

Fig. 11. 29 days. The chancres at the time of treatment.

Fig. 12. 2 weeks after treatment. The right chancre is healed, the left almost healed.

Fig. 13. 78 days after treatment. A recurrence in the form of multiple focal lesions which bear but little resemblance to the original chancres and only one of which could be regarded as a chancre recurrence.

PLATE 93.

Figs. 14 to 16. Recurrence of focal and diffuse lesions after healing of chancres.

Fig. 14. 43 days. The chancres at the time of treatment.

Fig. 15. 35 days after treatment. The chancres completely healed.

Fig. 16. 64 days after treatment. Recurrence of multiple nodules with diffuse infiltration of the scrotum.

Figs. 17 to 19. Recurrent papular lesions at the edge of the scar of a healed lesion.

Fig 17. 37 days. The chancres at the time of treatment.

Fig. 18. 21 days after treatment.

Fig. 19. 77 days after treatment. A thin, smooth scar with two small papules at its outer edge.

PLATE 94.

FIGS. 20 to 22. Healing of scrotal chancres with recurrence of chancre-like lesions entirely away from the site of the original lesions.

FIG. 20. 50 days. The original chancres.

FIG. 21. 7 days after treatment. Ulcers healed and chancres rapidly resolving.

FIG. 22. 133 days after treatment. Recurrent chancre-like lesion in the dorsal fold of the scrotum. The testicle is rotated towards the median line.

FIGS. 23 to 25. Transformation of a healing chancre into a diffuse infection of the scrotum followed later by multiple focal lesions of an erythematous character grouped mainly about the edges of the scar.

FIG. 23. 48 days. The time of treatment.

FIG. 24. 21 days after treatment. Chancres almost resolved but replaced by a diffuse infiltration of the scrotum. Note the thickened and refractile fold of the scrotum on the right.

FIG. 25. 35 days after treatment. Multiple focal lesions grouped particularly about the edges of the scars. Slight exfoliation.

PLATE 95.

FIGS. 26 to 28. Healing of scrotal chancres with recurrence in the head of the epididymis.

FIG. 26. 48 days. The chancres.

FIG. 27. 15 days after treatment. The chancres in process of healing.

FIG. 28. 30 days after treatment. The chancres healed; recurrent nodules in the head of the epididymis in both testicles.

FIGS. 29 to 31. Late recurrence of a minute papular lesion.

FIG. 29. 46 days. The chancres.

FIG. 30. 43 days after treatment. The chancres almost healed.

FIG. 31. 105 days after treatment. The recurrence. A minute point of infiltration in the scrotum, marked by the arrow.

PLATE 96.

FIGS. 32 to 37. Inguinal adenopathy associated with scrotal infection.

FIG. 32. Appearance of the inguinal region of a normal rabbit.

FIG. 33. 5 days. Acute swelling of inguinal nodes.

FIG. 34. 7 days. Acute swelling. Left, a single enlarged node; right, a large node situated over the cord and two smaller nodes at the edge of the ring, marked by arrows.

FIG. 35. 18 days. Enlargement with induration. Marked but unequal involvement of the nodes. Chancres small.

FIG. 36. 18 days. Marked lymphadenitis on the left with two small shotty nodes on the right, marked by arrows.

FIG. 37. 82 days. An asymmetrical involvement of the inguinal nodes. The enlarged node on the left is situated high in the groin.

PLATE 97.

FIGS. 38 to 43. Inguinal adenopathy associated with scrotal infection.

FIG. 38. 23 days.   Lymphadenitis of moderate degree.

FIG. 39. 28 days.   Indurated nodes.   Chancres small; nodes large and unequal.

FIG. 40. 64 days.   A flattened mass of small nodes on the right with a single rounded node on the left.   Chancres large; nodes moderately enlarged but intensely indurated.

FIG. 41. 60 days.   Extremely large chancres with apparently rather small lymph nodes.   The nodes are deep, however, and lie directly in the crease of the groin.

FIG. 42. Same as Fig. 41.   The left node picked up between the thumb and finger.

FIG. 43. 63 days.   Marked equal and symmetrical lymphadenitis with slight lesions of the scrotum.   Same animal as in Fig. 5.

PLATE 90.

(Brown and Pearce Experiment il syphilis in the rabbit   III)
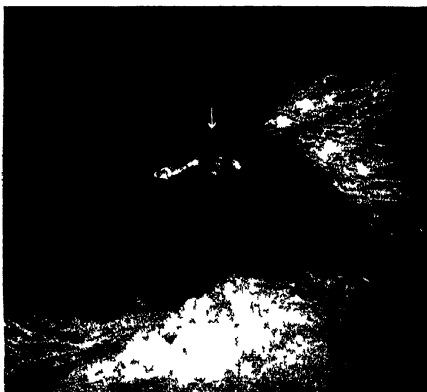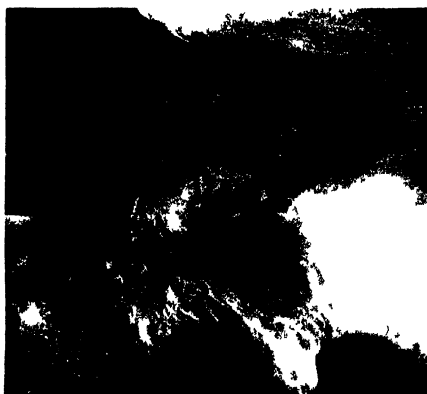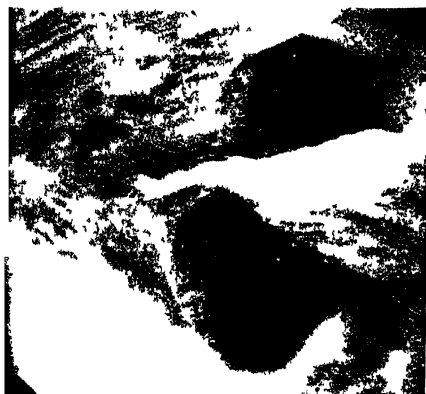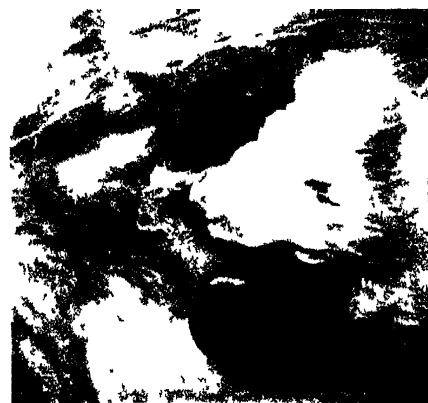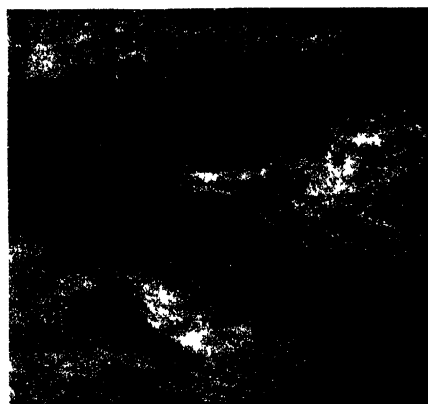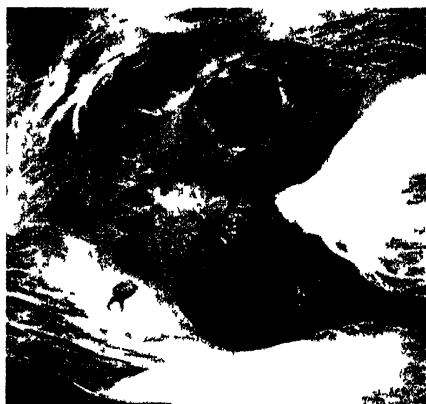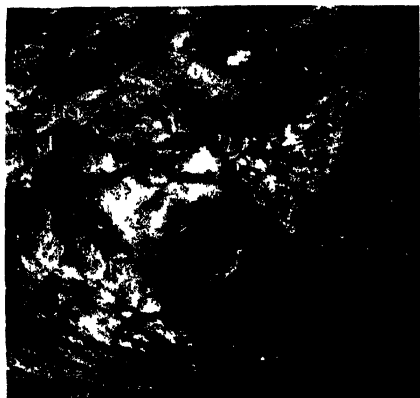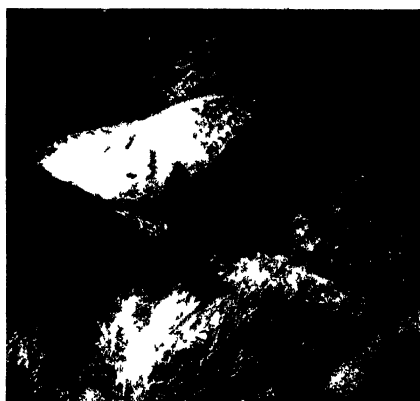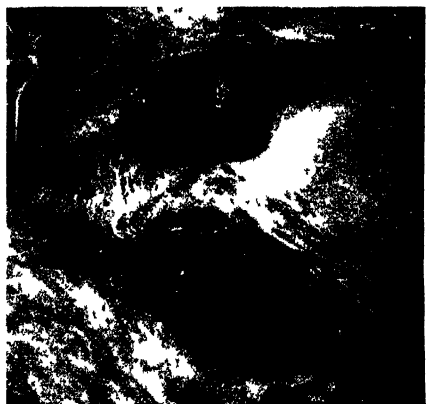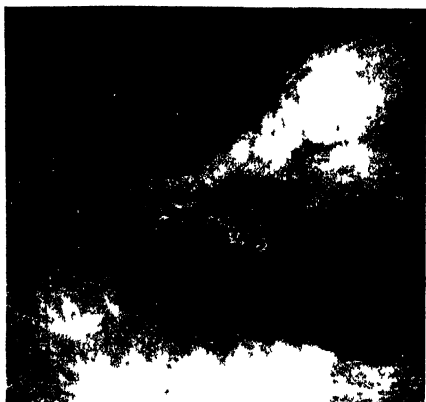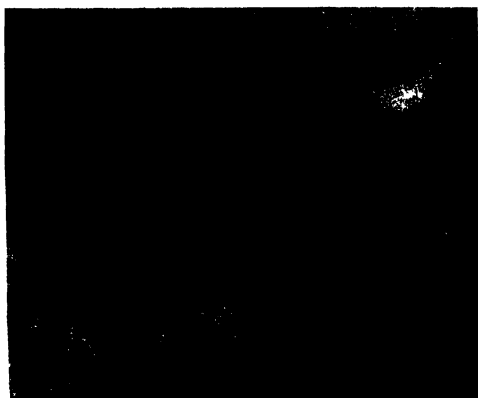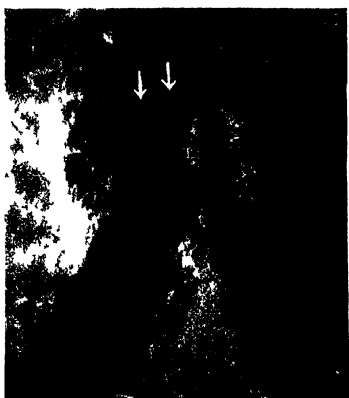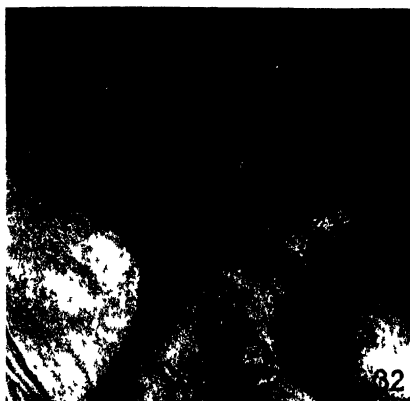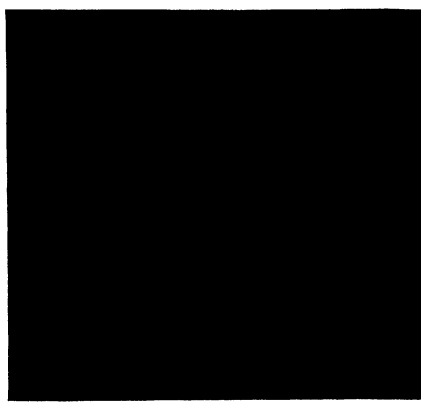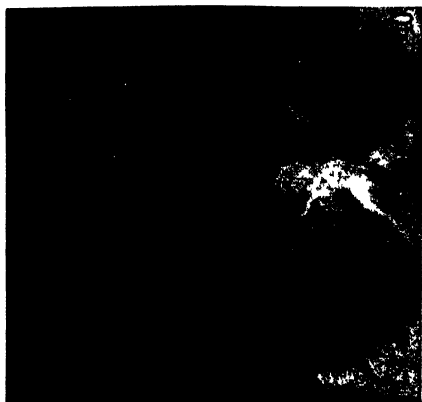
SEGMENTING TERTIAN MALARIAL PARASITES ON RED CORPUSCLES SHOWING LITTLE OR NO LOSS OF HEMOGLOBIN SUBSTANCE. EVIDENCE OF MIGRATION.*

By MARY R. LAWSON, M.D.

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PLATES 2 AND 3.

Migration of Malarial Parasites.

The malarial parasite has been described as attaching itself when young to a red corpuscle and remaining there during the entire period of its life cycle, the destruction of the infected corpuscle corresponding to the segmentation of the parasite. But this is not so. Each parasite destroys several red corpuscles, migrating to a fresh corpuscle as soon as one is destroyed. I have seen migrating parasites in eight different stages of development in tertian infections, the oldest form being the presegmenting parasite (Figs. 1 to 5).

Segmenting Parasites Attached to Red Corpuscles Whose Hemoglobin Is Intact or Nearly So (Figs. 6 to 49).

Thayer,[1] discussing tertian parasites, states: "In certain instances, however, sporulating forms may be found within corpuscles which are no larger than the normal red cell, and which are but little decolorized." He writes that Bastianelli and Bignami[2] are inclined to believe that such bodies are more common in cases of anticipating tertian fever. But Thayer states that his observations have not been sufficient to justify

[1] Thayer, W. S., Lectures on the malarial fevers, New York, 1897, 55.
[2] Bastianelli and Bignami, quoted by Thayer,[1] p. 55.

the formation of a definite opinion concerning this point. He makes no attempt to explain the occurrence; but he has pictured two segmenting tertian bodies on normal appearing red cells, designating them as instances of "precocious segmentation."[3]

It seems to me that the presence of segmenting parasites on red corpuscles with hemoglobin intact or nearly so is evidence that the parasites have recently attached themselves and have not had sufficient time to cause any appreciable damage to the infected cells and the pigment in connection with these segmenting parasites is additional evidence of their previous attachments to red corpuscles.

Infected Red Corpuscles of Tertian Infections.

One of the most striking features of tertian infections is the rapidity with which the parasites enlarge and decolorize the infected cells. Even the very young parasites do this soon after their attachment, and segmenting parasites are most frequently described as attached to decolorized cells. Marchiafava and Bignami[4] write: "one of the most characteristic properties of the tertian parasite is the rapidity with which the infected red cell becomes decolorized and swollen." The infected corpuscles in the accompanying plates show such a slight amount of alteration due to parasitic action that it is obvious that they could not have been occupied by the attached parasites for the entire period of their development.

While all observers have noted that the infected corpuscle is enlarged as a result of parasitic action, there are also instances in which it is enlarged as a result of the large size of the attached segmenting body. For instance, the segmenting bodies illustrated in Figs. 22 to 25 are larger than normal corpuscles at the same magnification, and they are too large to occupy corpuscles the size of those pictured in Figs. 7 to 10 without enlarging the corpuscles. The red corpuscles being more elastic than the parasites are readily enlarged when pressure is exerted. This mechanical enlargement of a corpuscle means a thinning of its substance, consequently it is usually paler than one

[3] Thayer,[1] Plate 1, Figs. 16 and 17.

[4] Marchiafava, E., and Bignami, A., Malaria, in Stedman, T. L., Twentieth century practice, New York, 1900, xix, 63.

of normal size. The increased size of many of the infected cells shown in the accompanying plates is the result of the large size of the attached parasites rather than of parasitic action.

Segmenting Bodies of Tertian Infections.

The tertian segmenting bodies are larger than those of the quartan or æstivo-autumnal infections. Within certain limits, from about 14 to 26, they show no fixed number of segments; occasionally I have seen fewer than 14, rarely more than 26. When more than 26 are seen it means, usually, that two segmenting bodies are attached to one corpuscle. It is interesting to note that the small segmenting body does not necessarily mean that the parasite has fewer segments. The pigment in connection with these bodies is usually collected in a mass, but it may be scattered among the spores. The young parasites resulting from the segmentation of these bodies attached to healthy appearing corpuscles frequently attach themselves independently and remain until the corpuscle is destroyed.

SUMMARY.

1. Malarial parasites destroy more than one red corpuscle, migrating to another as soon as one is destroyed.

2. Pigmented segmenting parasites attached to red corpuscles whose hemoglobin is intact or nearly so are *prima facie* evidence of migration.

3. Infected corpuscles in tertian infections may be enlarged as a result of the large size of the attached parasite as well as by parasitic action.

4. The young parasites resulting from the segmenting bodies attached to healthy red corpuscles usually attach themselves independently to the infected corpuscle and remain there until the corpuscle is destroyed.

EXPLANATION OF PLATES.

PLATE 2.

TERTIAN PARASITES.

Magnification × 1,807.

FIGS. 1 to 5. Presegmenting parasites free in the blood serum. These are parasites in the last free stage of migration.

FIG. 6. A presegmenting parasite attached to a red corpuscle which is enlarged but with hemoglobin nearly intact.

FIGS. 7 to 11. Segmenting parasites attached to red corpuscles which are practically normal both in size and hemoglobin content.

FIGS. 12 to 24. Segmenting parasites attached to red corpuscles whose hemoglobin is practically intact; the cells are enlarged slightly owing, in Figs. 22 to 24, to the large size of the attached parasites.

FIG. 25. A presegmenting parasite attached to a red corpuscle which is normal in hemoglobin content but enlarged by the large size of the parasite.

PLATE 3.

TERTIAN PARASITES.

Magnification × 1,807.

FIGS. 26 to 30. Presegmenting parasites attached to normal red corpuscles which are enlarged by the large size of the attached parasites.

FIGS. 31 to 33. Segmenting parasites attached to red corpuscles which show a slight alteration due to parasitic action. Note the fine stippling of the cells (Shüffner's granulation).

FIGS. 34 to 49. Segmenting parasites attached to fairly healthy red corpuscles. Note the large number of segments of some of the segmenting bodies.
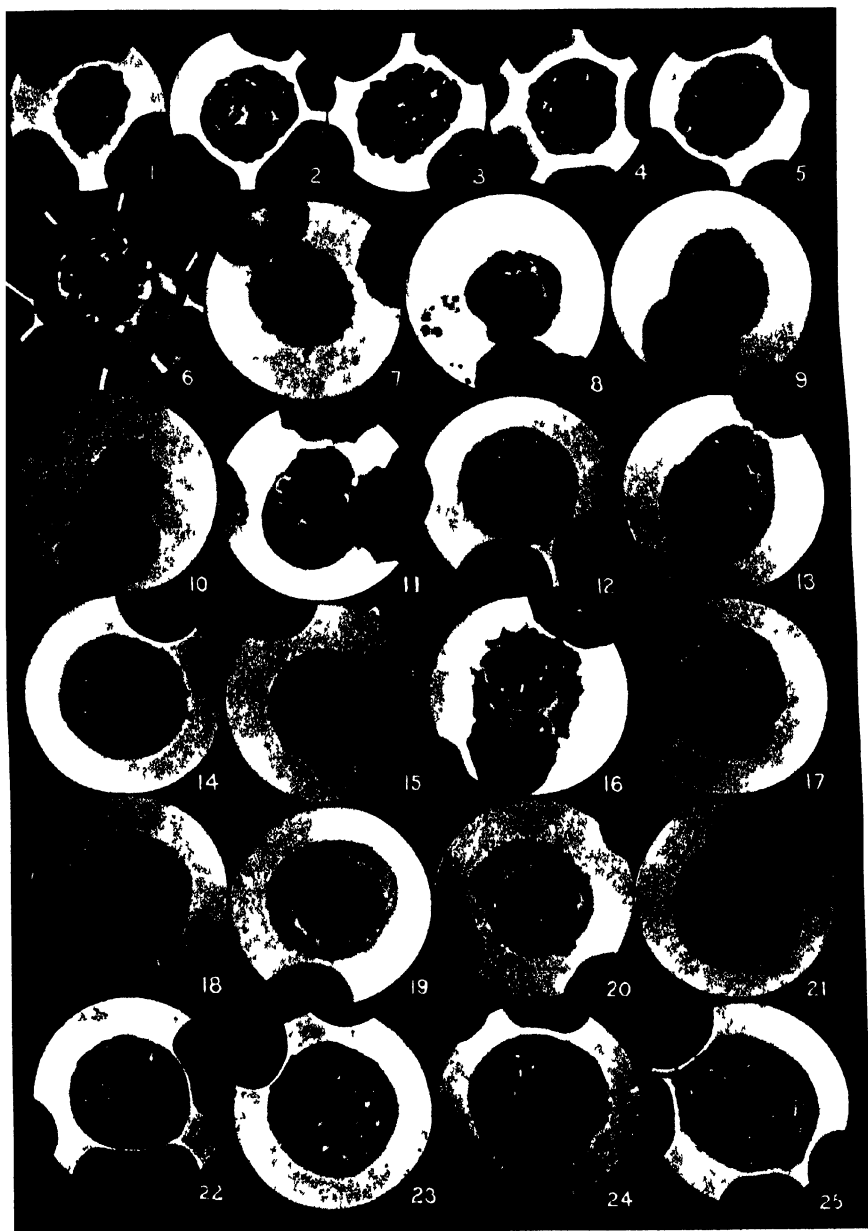
EXPERIMENTS ON THE PRODUCTION OF SPECIFIC ANTISERA FOR INFECTIONS OF UNKNOWN CAUSE.

III. NEPHROTOXINS: THEIR SPECIFICITY AS DEMONSTRATED BY THE METHOD OF SELECTIVE ABSORPTION.

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PLATES 5 TO 7.

The present study was undertaken as a continuation of work begun in this laboratory on the production of antisera for infections of unknown cause. It was found[1] that the infected tissues of animals might serve in some cases as an antigen for the production of immune sera, though with such antigens the sera inevitably contained antibodies injurious to the tissues of the animal. Experiments showed, however, that the sera can be largely deprived of these latter by incubation with successive portions of red cells without impairment of their protective value. Whether there are substances injurious to special organs which cannot so be taken out has remained to be determined. The problem is closely connected with that of the specificity of cytotoxins.

For most organs truly specific cytotoxins have not as yet been produced, despite many attempts. They have been elicited, though, for certain organs more or less isolated in the body. Zinsser sums up as follows on the subject:[2]

"Recent critical studies . . . . have revealed . . . . that the specificity of a serum produced with the tissues of one organ is not strictly limited to this organ alone, and that the serum may injure other organs as well. It is true, indeed, that there are certain cells and tissues in the body such as spermatozoa, the tissues of the testicles, the ovary, the lens of the eye, and possibly

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[1] Rous, P., Robertson, O. H., and Oliver, J., *J. Exp. Med.*, 1919, xxix, 283.

[2] Zinsser, H., Infection and resistance, New York, 1914, 92.

*the placenta, which have chemical characteristics so well defined and individual
that the cytotoxic sera induced by them have definite organ specificity. The
same to a more limited extent seems true of kidney substance (Pearce).* In most
cases, however, in which originally a specific cytotoxin was claimed, it has been
possible to show subsequently that the apparently selective injury was not due
to organ specificity alone but to the fact that the injection of tissue macerates,
even when sufficiently freed from blood, induced the formation of considerable
amounts of hemagglutinins and hemolysins."

In the light of such facts our problem resolves itself into a com-
paratively simple one: to determine whether one of the so called
specific cytotoxins can be removed from a serum by exhausting the
latter with red cells.

In selecting an organ for the study of "specific" cytotoxic sera the
kidney seemed best adapted, because injuries to it can be determined
by functional as well as histological methods.   Pearce[3] has brought
forward evidence that a serum can be produced which is, as he says,
at last "special" in its action on the kidney.   He leaves in doubt
whether it can be termed specific in the strict sense, for despite the
use of blood-free kidneys as antigen, his sera invariably contained
hemolysins and hemagglutinins which might possibly account for
their damaging effect on the kidney.

We have undertaken, first, to obtain a serum such as Pearce de-
scribes and observe its effects on the kidney; second, to determine
whether the principle injurious to the kidney can be removed from
this serum by absorption with red blood cells or with kidney tissue;
and, third, to compare the efficacy of serum exhausted with red cells
and that similarly treated with kidney tissue.

Production of Antikidney Serum.

Pearce's method was followed.[3]

Large dogs were etherized and under special precautions for asepsis their·
kidneys were removed and placed in glass boxes.   The neighboring portion of
the aorta was left attached to the kidneys in order to facilitate the passage of a
cannula into the renal artery; and by prolonged washing with sterile salt solu-
tion the kidneys were practically freed of·blood.   The capsules were then stripped,

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[3] Pearce, R. M., *J. Med. Research*, 1904, xii, 1.

the kidneys weighed and put through a meat grinder. The macerated tissue was pushed through a tea strainer of fine mesh with the aid of a pestle, small quantities of salt solution being added from time to time to facilitate its passage. The entire procedure was carried on under a glass plate surrounded by a canopy of sterile muslin which effectively protected the material from contamination.

The resulting heavy suspension in salt solution passed readily through a needle of medium sized bore. The material was usually injected into animals within 48 hours of its preparation, though occasionally it was kept in a frozen condition for some days prior to use. A number of rabbits received three to five intraperitoneal injections, each of from 4 to 8 gm. of kidney tissue, at intervals of 7 days, and they were bled on the 10th day after the last one. The sera were inactivated (56°C. for $\frac{1}{2}$ hour) prior to use and their hemolytic and hemagglutinating titers tested, and cultures taken.

The sera without exception possessed moderately strong hemagglutinins and weak hemolysins for dog cells. Tests were made in the usual manner with graded dilutions in salt solution. Equal portions of the serum dilutions and of a 5 per cent suspension of thrice washed dog cells were used in the agglutination tests, and for hemolysis guinea pig complement in a dilution of 1 to 10 was added.

Precipitins were tested for but when found were negligible in amount.

Effects of Antikidney Serum.

Nine dogs were given a single intravenous injection of inactivated antikidney serum. The dose was 1 to 2 cc. per kilo of body weight. In most cases extremely marked renal changes resulted, differing considerably from those described by Pearce.

Prior to injection the general condition as well as the urine of the animals was carefully followed for a period of at least 2 weeks. Many supposedly normal dogs show albumin and casts in the urine without evident cause, and much delay was often experienced in finding animals suitable for our purpose. Within 24 to 48 hours after injection the animals appeared rather lethargic and gave the general impression of being sick. This period of depression passed off completely, or persisted, according to the potency of the serum and corresponding in a general way to the damage sustained by the kidney. The changes in the urine were marked. Severe albuminuria rapidly developed, with many casts, mostly granular at first but later many of them hyaline and occasionally waxy. This always occurred within 48 hours after the injection of the serum and persisted for the few days before the dog was killed. A trace of blood was sometimes noted.

With the exception of one dog (Dog C), which was observed for 4 months, all of the dogs were chloroformed and autopsied 10 to 14 days after injection. The kidneys were removed just prior to death or immediately after it. The viscera were examined, but no lesions were ever found that could be attributed to the serum. On two occasions more than 750 cc. of clear straw-colored fluid were present in the peritoneal cavity. No general edema was ever observed.

The kidneys showed pathological changes practically always, often of marked degree. In typical cases the organs were enlarged, and on stripping the capsule the kidney surface was everywhere mottled with bright red dots, from 0.1 to 1 mm. in diameter (Fig. 1, *A*). On section these were found to be the surface indices of hemorrhages into the cortex, the most characteristic gross finding. The hemorrhages were in general wedge-shaped, with the base of the wedge at the kidney surface. The glomeruli were indistinct and apparently had no relation to the hemorrhages. The cortex was swollen and opaque, and the pale medulla occasionally showed traces of hemorrhage extending from the cortex.

Histologically glomerular lesions, not the hemorrhages, were the most marked as well as constant finding. They occurred without hemorrhages when the serum was weak. In well marked instances there was an extensive necrosis in the glomerular tufts followed by obliterative hyaline changes (Figs. 2 and 3). A large proportion of the glomeruli were thus affected, though in even the most pronounced instances a sufficient number remained for kidney function. Accompanying the early cell changes was a plugging of the glomerular capillaries with fibrin and often a fibrinous exudation into the capsular space (Fig. 4). Desquamation and proliferation of the cells of the loops with occasional mitotic figures were observed. The end-result was often a complete obliteration of the glomerulus.

According to Pearce the most striking changes occur in the tubules. In our experiments lesions there were usually not pronounced, though sometimes marked cloudy swelling and desquamation were observed.

Next to the glomerular changes already described the most frequent findings were casts and hemorrhage into the tubules (Figs. 2 and 4). When the serum was strong small groups of a dozen to twenty tubules, usually in the neighborhood of a glomerulus, had their lumina filled

with blood cells (Fig. 5). These were the punctate hemorrhages so prominent in the gross specimen (Fig. 1).

There was also observable sometimes a considerable round cell infiltration between the tubules, especially in the neighborhood of the glomeruli. Some of the destroyed glomeruli showed a very heavy infiltration with lymphocytes and polymorphonuclear leucocytes (Fig. 6).

As a control two dogs were given large doses of normal rabbit serum. The results were negative as regards changes in the kidneys.

Effect of Antikidney Serum Exhausted with Red Cells.

The sera which caused the lesions described above contained, as already stated, hemolysins and hemagglutinins, though these were never strong. Steps were now taken to determine whether the action of the serum was due to them. Pearce performed a single experiment of the sort, submitting serum to contact with washed dog red cells for 1 hour at 0°C., and he found it thereafter "only faintly hemolytic." Kidney lesions were still produced. He records no observations, however, on hemagglutinins in the serum, which are often stronger and persist longer than hemolysins. In view of their ability to cause liver necroses,[3] such antibodies might well have been responsible for the lesions occurring in the kidneys.

By repeated absorptions with washed dog red blood cells, the technique of which has been previously described in detail,[1] our sera were completely deprived of hemolysins and hemagglutinins.

The results of the injection of dogs with serum thus treated are contrasted in Table I with findings in control animals receiving injections of corresponding doses of the same serum, untreated.

As the table shows, the exhaustion of the serum with red cells does not affect its ability to cause urinary changes. The albuminuria and cast formation were slightly less marked than in the controls. On the other hand, there were to the naked eye striking gross differences in the kidneys of the two series. It has been said that the untreated serum causes punctate hemorrhages in the renal cortex, often so numerous as to mottle diffusely the kidney surface. They are almost, or completely, lacking in animals given exhausted serum

TABLE I.

Effects of Nephrotoxic Sera, Untreated and Exhausted with Red Cells.

Experiment No.	Dog.	Serum No.	Treatment.	Serum titer. Action disappears at dilution of.	Dose per kilo of animal.	Effect on urine.		Killed after.	Condition of kidneys.
						Albumin.	Casts.		
					cc.			*days*	
1	A	1*	Untreated.	1/256 lysis. 1/128 agglutination.	1.55	+−	++	8	Slight gross changes; characteristic glomerular lesions and microscopic hemorrhages. Casts.
	Aa		11 cc. of serum underwent four absorptions with 16 cc. of red blood cells in all.	No lysins or agglutinins.	1.59	−	+	8	A few casts in tubules. Marked epithelial lesions.
2	B	2*	Untreated.	1/256 lysis. 1/128 agglutination.	1.33	−	+	8	Marked epithelial lesions. Casts.
	Ba		13.5 cc. of serum underwent four absorptions with 16 cc. of red blood cells.	No lysins or agglutinins.	1.72	+	−	8	Moderate epithelial lesions.
3	C	3	Untreated.	1/4 lysis. 1/1,024 agglutination.	1.65	++	+++	119	Few fibrotic glomeruli. No increase of interstitial tissue.
	Ca		17 cc. of serum underwent five absorptions with 25 cc. of red blood cells.	No lysins or agglutinins.	1.60	+++	+++	19	Severe glomerular lesions and hemorrhages. Many casts.

4	D	5	Untreated.	1/10 lysis. 1/30 agglutination.	0.60	++	++	9	Moderate glomerular lesions and hemorrhages. Many casts.
	Da	5	37 cc. of serum underwent five absorptions with 17 cc. of red blood cells.	No lysins or agglutinins.	1.36	++	+	9	Changes definite, but less than in the control.
5	E	5	Untreated.	As in Experiment 4.	1.85	++	++	10	Marked glomerular lesions. Hemorrhages and casts.
	Ea	5	Absorbed as in Experiment 4.	As in Experiment 4.	1.86	++	+	10	As in control, but slightly less marked.
6	F	6	Untreated.	1/4 lysis. 1/512 agglutination.	1.66	++	++	11	Numerous hemorrhages and glomerular lesions. Many casts.
	Fa	6	22 cc. of serum underwent seven absorptions with 22 cc. of red blood cells.	No lysins or agglutinins.	1.85	++	++	11	As in control.
7	G	7	Untreated.	1/128 lysis. 1/128 agglutination.	1.29	+++	++	10	Moderate changes of characteristic sort.
	Ga	7	15 cc. of serum underwent eleven absorptions with 34.5 cc. of red blood cells.	No lysins or agglutinins.	1.00	+++	++	10	As in control, but slightly fewer glomerular lesions.
8	H	4	Untreated.	1/128 lysis. 1/128 agglutination.	0.65	++	++	11	Moderate changes of characteristic sort.
	I	4	"	1/128 lysis. 1/16 agglutination.	1.65	+++	+++	22	Characteristic but moderate lesions.

* Sera 1 and 2 were kept 3 months before their absorption and the animal tests, which may explain the mildness of their

(Fig. 1, *B*).   To judge from the gross appearance one would suppose that exhaustion had entirely deprived the serum of its ability to cause renal lesions.   Microscopically, however, it is seen that all of the lesions previously described are present save the hemorrhages. The glomerular and tubular lesions are in some instances less marked than in the controls, but in other cases they are equally well defined.

Type Experiment 1.   Action of Nephrotoxic Serum.   (a) Untreated; (b)Absorbed with Red Cells.—(*a*) Dog F, male, weight 6 kilos, after 2 weeks observation, during which time the urine was frequently examined and found to be free of casts and albumin, was given, on Mar. 26, 1918, 10 cc. of Serum 6 intravenously.   The dog behaved normally after the injection.   (This serum was prepared by injecting Rabbits 1 and 2 with blood-free dog kidney suspension.   On Oct. 1, 1917, the suspension contained 4 gm. of kidney tissue; Oct. 8, 6 gm.; Oct. 15, 8 gm.; Oct. 22, 5 gm.   Oct. 30.   The rabbits were bled to death and the serum preserved in the ice box.   On Mar. 22 the two sera were pooled and the titer was determined. Hemolysis disappeared at a dilution of 1 to 4 and agglutination at 1 in 512. Inactivation was done at 56°C. for ½ hour.)

Mar. 27.   Urine very dark amber, alkaline, cloudy.   Dense ring of albumin with nitric acid test.   Many epithelial cells, a few red blood cells and leucocytes. No casts.   Guaiac test positive.   Mar. 28.   Urine shows a great deal of albumin.   Guaiac test positive.   Occasional granular casts.   Mar. 30.   Same findings.   Apr. 1.   Albumin present.   Guaiac test negative.   Very many hyaline and granular casts.   Apr. 3, 5, and 6 showed the same findings.

On Apr. 6 the dog was etherized and an autopsy performed, with negative findings in all the organs except the kidneys, which were swollen and congested.   On stripping the capsule innumerable fine red dots up to 1 mm. in diameter were revealed on the surface of the kidney (Fig. 1, *A*).   On section the kidneys were swollen and opaque, the cortex showing a definite widening.

Histological examination showed innumerable punctate cortical hemorrhages into the tubules, filling the lumina of scattered groups.   There were a few interstitial hemorrhages.   The majority of the glomeruli showed marked changes. Many of the cells in the tufts were necrotic, and numerous coils were plugged with fibrin.   Mitoses were not infrequent.   There was some interstitial round cell infiltration here and there between the tubules, especially in the neighborhood of the glomeruli.   Many casts were seen in the tubules, but only slight changes in their epithelium.

(*b*) Dog Fa; weight 5.25 kilos.   Observed 2 weeks, during which the urine was free from albumin and casts.   The animal was given intravenously, on Mar. 26, 1918, 9.8 cc. of Serum 6, which had been absorbed six times with washed dog red blood cells.   (The total amount of serum submitted to absorption was 22 cc.   3 cc. of washed dog red cells were added to it, the tube inverted several times and incubated at 37°C. for 1 hour.   Dense agglutination resulted.   After centrifu-

gation the serum was pipetted into another tube, another 3 cc. of washed cells were added, and incubation was repeated. In all, seven such absorptions were carried out on this serum, after which it was tested for hemolysins and agglutinins and found to possess none. Cultures taken prior to its use for injection proved that it was sterile.)

Mar. 27. Urine showed a trace of albumin, no casts. Guaiac test positive. Mar. 28. Same findings. Occasional granular casts. Mar. 30. Large amount of albumin, many granular casts, no blood. Apr. 3, 5, and 6. Same findings. Apr. 6. The dog was etherized and all organs found to be normal except the kidneys, which were large and pale. The capsules were stripped and revealed a few pin-point hemorrhages, probably one for every fifty seen in the case of Dog F (Fig. 1, *B*).

Histological examination revealed changes almost identical with those observed in the case of Dog F, except that there were fewer hemorrhages (Fig. 2).

Effect of Ordinary Hemolytic-Hemagglutinative Serum.

In view of the fact that hemolysins have far more effect *in vivo* than *in vitro*,[4] it seemed necessary to test the effect on the kidney of an ordinary hemolytic and hemagglutinating serum produced by the injection of dog red cells. For it might be contended, as explaining the injurious effect on the kidney of our exhausted serum, that such serum still contained traces of hemolysin and hemagglutinin, which, while not demonstrable *in vitro*, were active *in vivo*. Rabbits were immunized therefore against washed dog red cells and an anti-dog serum of high agglutinin and hemolytic titer was produced. This was inactivated and injected intravenously into dogs, a part of it after absorption with dog red cells.

Type Experiment 2. Effect of Hemolytic and Hemagglutinating Serum. (a) *Untreated; (b) Absorbed with Red Cells.*—The serum used was prepared by injecting rabbits intraperitoneally with a 20 per cent suspension of thrice washed dog cells in salt solution. Five injections were made at 7 day intervals, and the rabbits were bled on the 8th day following the last injection. The resulting sera were pooled and found to be hemolytic in dilutions up to 1 in 256 and agglutinative in dilutions up to 1 in 1,025. A portion of the pooled serum was subjected to eleven absorptions with dog red cells and again tested, with the result that no hemolysins or agglutinins were found. In detail the method of absorption was as follows:

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[4] Muir, R., and M'Nee, J. W., *J. Path. and Bacteriol.*, 1911–12, xvi, 410.

Absorption.	Amount of serum.	Dog red cells.	Incubation.
	cc.	*cc.*	*min.*
1	20	3	30
2		8	30
3		3.5	40
4–11		4 each.	60–120

(a) Dog R, weight 5.5 kilos, was given, on May 6, 1918, 2 cc. of the untreated portion of the serum. May 8. The urine, previously normal, is now smoky in appearance, containing a trace of albumin and much hemoglobin but no casts. May 10. Urine dark red; faint trace of albumin; many granular casts containing brownish pigment. Tests for blood and bile positive. May 14. Urine dark amber; albumin negative; no casts. Bile pigment and blood present. May 17. Urine amber, no albumin, hemoglobin, or casts. May 18. Dog etherized and kidneys removed. Histological examination showed much brown, granular pigment in the spleen and liver. The kidneys were entirely normal except for widespread, fairly abundant, light brown pigment granules in the cells of the proximal convoluted tubules.

(b) Dog S, weight 5.25 kilos, was given intravenously on May 2, 1918, 12 cc. of the absorbed portion of the serum. Immediately after the injection dog vomited, was prostrated, and passed feces. Complete recovery took place in 1 hour. The urine was examined on May 3, 4, 6, 8, 10, and 14, but on none of these occasions were any abnormal constituents found. On May 14 the dog was etherized. Autopsy showed all organs normal. On histological examination the kidneys were normal.

It will be seen that the unabsorbed serum produced no important anatomical changes in the kidney despite the marked blood destruction it caused. Pearce, using such a serum, reports a fatty condition limited almost entirely to the loops of Henle. Otherwise the urinary and kidney findings which he reports in animals that survived a few days were the same as here reported. He did not try absorption, which, as we found, renders the serum innocuous to the blood as well as the kidneys. Altogether the facts warrant the conclusion that the changes produced by an antikidney serum exhausted with red cells cannot be attributed to persisting hemolysins or agglutinins. It is interesting that the most striking gross lesion caused by the antikidney serum, namely the punctate cortical hemorrhages, is not produced by an anti-red-cell-serum, although absorption with red cells deprives the antikidney serum of its ability to produce such a lesion.

Effects of the Exhaustion of the Serum with Kidney Tissue.

Can the injurious principle in the antikidney serum be removed by absorption with kidney tissue? This point was now investigated.

For the purpose of proper absorption with kidney tissue it was obviously necessary that the latter should be in as finely divided a state as possible, so as nearly to resemble in amount of absorbing surface a red cell suspension. Coarse fragments of tissue would offer relatively little surface and might be expected to yield poor results.

To obtain a fine suspension the tissues of washed kidneys of dogs were ground in the way already described, taken up in normal salt solution, and shaken with broken glass for 1 hour. The glass and larger tissue fragments were then removed by slow centrifugation, leaving a dense, milky suspension of finely divided material. On microscopic examination many free kidney cells were found in this fluid, which was now sedimented at high speed, and the sediment repeatedly washed by centrifugation until the supernatant fluid came away perfectly clear. The sediment was found to consist of parenchymal and other cells and fragments of them. For the purpose of absorption it was measured in bulk and used in suspension in the same manner as blood cells. It went into suspension readily.

Type Experiment 3 (Table II). Inactivated Antikidney Serum X Was Divided into three Portions: (a) Untreated; (b) Absorbed with Red Blood Cells; (c) Absorbed with Different Amounts of Kidney Tissue.—The untreated serum weakly agglutinated dog red cells in a dilution of 1 to 32 and hemolyzed them completely in a dilution of 1 to 4.

(a) *Results with Untreated Serum.*—Dog U, weight 7 kilos, with a urine negative for albumin and casts, received 1.35 cc. per kilo of the untreated serum on June 11, 1918. No symptoms following the injection. The urine on June 14, 19, 21, and 22 showed a heavy cloud of albumin and many hyaline and granular casts.

On June 22 the dog was etherized and an immediate autopsy performed All organs were negative except the kidneys, which were swollen and congested. A few punctate hemorrhages were seen on the surface.

Histological Examination.—All organs negative except the kidneys, which showed characteristic changes. Many of the glomeruli were greatly damaged, though not a majority. All showed increase of cell nuclei, and the loops were often difficult to distinguish and appeared collapsed, apparently as a result of small occluding fibrin masses. There were some glomerular adhesions. The proximal convoluted tubules were definitely swollen, with much albuminous debris in the lumina, as well as many casts. The lumina of the descending portion of the loop of Henle were filled with red cells in places. There were small, scattered. areas of edema of the interstitial tissues. The liver and spleen showed nothing abnormal.

(*b*) *Results with Serum Absorbed with Red Cells.*—Dog V, weight 8.5 kilos, received intravenously, on June 11, 1918, 1.28 cc. per kilo of Serum X, absorbed with dog red blood cells. The following was the method of absorption.

Absorption.	Amount of serum.	Dog red cells.	Incubation.
	cc.	*cc.*	*min.*
1	22	6	50
2		3.5	60
3–4		3 each.	60 and 90

After the absorptions there were no demonstrable hemolysins or agglutinins in this serum.) No symptoms. Urine negative.

June 13. Urine negative. June 14. Albumin, dense ring. No casts or blood. June 19 and 21. Urine showed much albumin, few granular and hyaline casts. On June 21 the dog was etherized. All organs were found negative with the exception of the kidneys, which were congested and swollen; no surface hemorrhages.

Histological Examination.—All organs negative with the exception of the kidneys, which showed a few interstitial hemorrhages. The usual glomerular changes were present. Few casts seen in the tubules. Findings like those of Dog U but not quite so pronounced.

Dog W, weight 4 kilos, received intravenously, on July 13, 1918, 4.4 cc. of Serum X, absorbed with red cells as above. July 15. Urine contained much albumin, occasional granular casts, no blood. July 17, 20, 22, and 24. The urine contained much albumin, many granular casts, no blood. Dog etherized and kidneys removed on July 24. Autopsy showed all organs normal with the exception of the kidneys, which were large and pale, with a very few punctate hemorrhages on their surface.

Histological Examination.—The general picture was the same as that of Dog U.

(*c*) *Results with Serum Absorbed with Kidney Cells.*—Dog X, weight 6.25 kilos, received, on June 11, 1918, 8 cc. of Serum X, absorbed three times with kidney cells in exactly the same proportion and for the same period employed with red cells as above stated. No symptoms. Urine examined June 13, 14, 19, 21, and 22. With the exception of June 14, when a very slight trace of albumin was observed, it was negative throughout. Etherization and autopsy on June 22. All organs normal by gross and histological examination.

Dog Y, weight 5.75 kilos, received, July 13, 1918, 8 cc. of Serum X, which had been absorbed three times with washed kidney cells of dogs as just described. No symptoms. Urine showed slight traces of albumin and occasional granular casts on July 15 and 17. On July 20, 22, and 24 it was negative. July 24. The dog was etherized and autopsied. Kidneys small, slightly congested, otherwise normal. All other organs normal.

Histological Examination.—Negative.

Dog Z, weight 4.50 kilos, received, on June 27, 1918, 2.2 cc. per kilo of Serum X, absorbed with kidney tissue as described. There followed a severe reaction, with vomiting, rapid respiration, and passage of feces and urine. The general picture suggested an anaphylactic crisis. After 20 minutes there was a gradual cessation of these symptoms, with complete recovery.

July 1. General condition of dog good. Urine showed albumin and a few granular casts but no blood. July 3. Urine about the same. July 5. Many casts.

TABLE II.

Relative Effects of Untreated Antikidney Serum and Serum Exhausted with Red Cells and with Kidney Cells (Type Experiment 3).

Dog.	Serum X.	Total bulk of sediment used for exhaustion per cc. of serum.	Dose of serum per kilo of animal.	Urine changes.	Kidney changes.
		cc.	*cc.*		
U	Untreated.		1.35	++	Moderate lesions in epithelium and glomeruli.
V	Four absorptions with red blood cells. Total incubation 5 hrs.	0.70	1.28	++	Slight glomerular lesions; moderate epithelial.
W	" "	0.70	1.10	++	Moderate glomerular lesions; no hemorrhages. Many casts.
Q	" "	0.70	2.17	++	Severe glomerular lesions; epithelial injury. Casts.
X	Three absorptions with kidney cells. Incubation 4 hrs.	0.50	1.26	0	Occasional cast. Otherwise negative.
Y	" "	0.68	1.39	0	Occasional cast.
Z	" "	1.00	2.2	+	Rare glomerular lesion. Few casts.

July 8. Only a trace of albumin, few casts. Dog etherized and autopsied. General examination negative. Kidneys showed no gross changes except a few hemorrhages, very few compared with control.

Histological Examination.—Occasional glomerular changes of the sort already described, but on the whole most of the glomeruli were in excellent condition. There were not infrequent characteristic hemorrhages into groups of tubules, as well as interstitial hemorrhages. Tubules generally normal, with the exception of a few casts in lumina. Liver and spleen normal.

The results of the experiment are summarized in Table II.

DISCUSSION.

Pearce's conclusion that an anti-dog rabbit serum could be produced which has a special action on the kidney has been confirmed. He, however, left the question of the true specificity of such a serum in doubt, and we must now consider whether our experiments throw any light on the subject.

An element in antikidney serum causing a very striking lesion, (abundant punctate cortical hemorrhages) is removed from the serum by the exhaustion of the latter with red cells, although an ordinary anti-red-cell-serum fails to produce such lesions. But the principle most injurious to the kidney cannot be so removed, even when the number of absorptions and the total bulk of red cells are very large. Moreover, a serum of high titer obtained by immunization with washed red cells fails to produce kidney lesions in any way resembling those of the nephrotoxic serum, which is further evidence that the injurious principle of the latter is not an hemagglutinin or hemolysin. The absorption of the antikidney serum with kidney tissue removes the injurious antibodies, even when the amount of tissue employed, and presumably the absorbing surface, are much less than were used in similar absorptions with red blood cells. Illustrations of the point are to be found in Table II.

The criticism may be made that the great absorptive power of the kidney tissue as thus demonstrated need not be the consequence of specificity but may be related to physical conditions that obtain in the emulsion, which make its absorptive power greater than that of a red cell emulsion. The experiments of certain observers on the absorption of antibodies with substances which obviously have no specific relation to them, kaolin, for instance, might here be cited. This criticism, however, is primarily directed not at the specificity of the nephrotoxin, with which we are alone concerned, but at the basic theory of the specificity of antibodies in general.

The effects of the nephrotoxic serum are exerted on the glomeruli and to a somewhat less degree on the tubules. It may be asked whether a vascular lesion will not account for all of the results. The glomerular changes are not improbably secondary to an occlusion of the coils following injury to the vascular endothelium. Our ani-

mals were autopsied too late to furnish conclusive evidence on the point. But whether or not the primary lesion is vascular, we are certain at least that the kidneys alone are affected. Were this the result of a non-specific endotheliolysin other organs should serve equally as well as the kidney for an antigen because of their content in endothelial cells. Pearce, however, has shown that a serum produced by the use of liver tissue as antigen has no effect on the kidneys.

As has been stated, our investigation was begun with a view to determining whether an antiserum produced as a result of the injection of tissues of an infected organ can be freed of injurious tissue antibodies by its exhaustion with red cells. As far as the kidney is concerned the question has been answered in the negative. A serum produced by immunization with kidney tissue should be absorbed with material from this organ. When that has been done it is no longer injurious to the kidney. Whether these principles are of general application may be doubted, for it is admitted that the kidney constitutes a special case. Notwithstanding numerous attempts by various investigators, sera specific for the spleen, pancreas, and liver have never been conclusively demonstrated. To obviate all possibility of injury from specific antibodies, however, a serum produced by the use of a tissue antigen should be exhausted with tissue of an identical sort prior to its introduction into the animal body.

SUMMARY.

As Pearce has shown, a serum highly injurious to the kidney of dogs can be produced by the immunization of rabbits with washed renal tissue of the dog. The histological findings are striking and characteristic, the most noteworthy being a glomerular lesion of special type. The renal changes differ much from those Pearce described.

The injury to the kidney is not to be explained by hemolytic and hemagglutinative elements in the serum. The complete removal of such antibodies by exhaustion of the serum with successive portions of red cells fails to lessen materially its ability to cause kidney lesions. Furthermore, an ordinary hemolytic and hemagglutinative serum produced by the use of washed red cells as antigen fails to cause similar lesions.

The distinctive, injurious principle of antikidney serum can be removed and the latter rendered innocuous by absorption with kidney tissue. To all practical intents and purposes it would seem that nephrotoxic serum of the sort here described is specific.

If infected tissue is to be utilized as an antigen for the production of therapeutic antisera the latter must in some instances be exhausted with tissue of the same sort prior to introduction into the animal body.

EXPLANATION OF PLATES.

PLATE 5.

FIG. 1. Photograph of the kidneys of Dogs F and Fa, Type Experiment 1, Table I. (*A*) The first mentioned animal received untreated antikidney rabbit serum, and its kidney shows innumerable cortical hemorrhages. (*B*) The other received an even larger amount of the same serum, from which the hemolysins and hemagglutinins had been removed by repeated absorptions with dog red cells. Though the kidney shows almost no hemorrhages, it was found microscopically to be badly damaged (Fig. 2).

FIG. 2. Section of the kidney pictured in Fig. 1 of Dog Fa, Type Experiment 1. Three glomeruli are seen, all of which show necroses of varying size. The tubules are dilated and filled with hyaline casts. There is beginning round cell infiltration in the neighborhood of the glomeruli. Bausch and Lomb, obj. ⅛, oc. 1.

PLATE 6.

FIG. 3. Section from Dog Q, Type Experiment 3, which received antikidney serum absorbed with red cells. High power view of a necrotic glomerulus. Half of the glomerulus is entirely destroyed, and the other half severely compressed by the necrotic mass. There is a beginning invasion of the dead material by proliferating capsular cells. Bausch and Lomb obj. ⅛, oc. 1.

FIG. 4. Same animal. Weigert fibrin stain of an injured glomerulus. The necrotic area contains a large thrombus of fibrin. Three tubules are also seen filled with hyaline casts which take the fibrin stain. Bausch and Lomb obj. ⅛, oc. 1.

PLATE 7.

FIG. 5. Same animal. A group of tubules, lying just beneath the capsule, their lumina filled with red blood cells. Other tubules contain hyaline casts. Bausch and Lomb obj. ⅛, oc. 1.

FIG. 6. Same animal. An almost completely destroyed glomerulus. There is a marked infiltration of the glomerulus and the surrounding area with leucocytes, both polymorphonuclear and lymphocytic. Bausch and Lomb obj. ⅛, oc. 1.
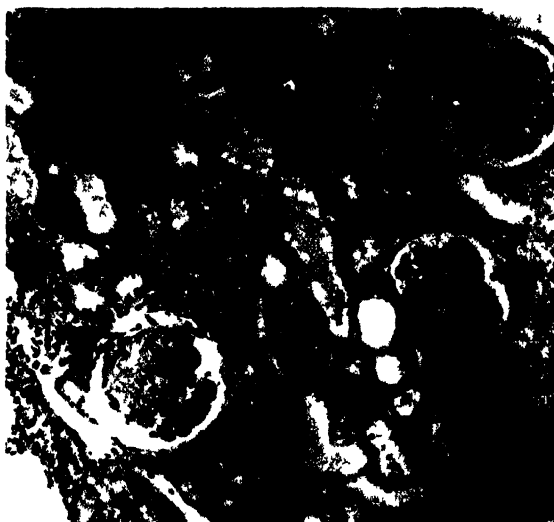
A   B

Fig. 1

Fig. 2.

FIG 3

FIG. 4

FIG. 5
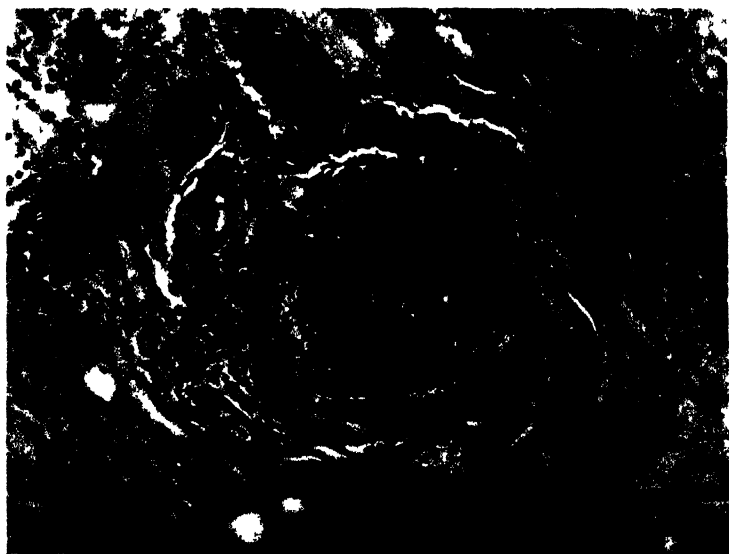
FIG. 6.

THE BILIARY FACTOR IN LIVER LESIONS.

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PLATES 17 TO 22.

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The qualification "biliary" has long been applied to a diverse group of hepatic lesions distinguished by a peculiar cirrhosis with more or less evidence of stasis in the finer bile ducts and inflammation of their walls. The actual part played by bile in the production of the connective tissue changes here seen and of chronic lesions in general of the human liver is not definitely known. According to some authors human bile is incapable of causing any permanent hepatic injury. If this be true man differs from all other well studied animals, for in them without exception aseptic bile stasis results in important local changes. There is positive evidence, too, that human bile may on occasion be the cause of important damage to the liver. Sudden total obstruction of the common duct results in the so called icteric necroses,[1] which may attain to the size and character of "biliary infarcts,"[2] large wedge-shaped areas of bile-stained necrosis such as regularly follow obstruction in rabbits and guinea pigs. Long continued stasis, when uncomplicated by infection, as is rarely the case, has been known to result in stellate cirrhosis with many new-formed bile ducts, a lesion found in the rabbit under like circumstances;[3] and, as in this animal, complete local stasis leads to liver atrophy.[4] Further examples might be cited. For the purposes of the present work we shall assume that human bile, while innocuous as compared with that of certain other species, can produce liver injury. Our

[1] Eppinger, H., *Beitr. path. Anat. u. allg. Path.*, 1902, xxxi, 230.

[2] Carnot, P., and Hervier, P., *Arch. méd. exp. et anat. path.*, 1907, xix, 76.

[3] Rolleston, H. D., Diseases of the liver, London, 2nd edition, 1912.

[4] Brissaud, E., and Sabourin, C., *Arch. physiol. norm. et path.*, 1884, iii, series 3, 345.

aim has been to obtain through experiment a better understanding of "biliary" lesions with special reference to the share of bile in their causation. No attempt will be made to ignore the factor of infection, which without doubt has for many instances a prime importance.

Choice of Animals.

The differences in the commoner laboratory animals as regards amount of the bile and its local effects have been admirably set forth by Quincke and Hoppe-Seyler.[5] The years since their summary have added little that is new. The rabbit and guinea pig respectively secrete nearly seven and eight times as much bile as the dog, and ten and twelve times as much as the cat. Man's rate of secretion places him near to the last mentioned animals. In them the constitutional effects of total biliary obstruction are more marked than the local, while in man death usually occurs of toxemia before cirrhotic changes are manifest.[5] In the guinea pig and rabbit, on the contrary, closure of the common duct results in fulminant liver destruction with cirrhosis, and the death of the animal, which follows after a few weeks at most, is traceable to this cause. The development of lesions following local duct obstruction is far more rapid than in the dog and cat, changes that may take a twelve-month in these latter[6] requiring only a fortnight or less in the rabbit. And in the rabbit, furthermore, secondary infection occurs but rarely, whereas in the dog it is so frequent a confusing factor as to have balked many investigators. On the basis of recent experience we have no hesitation in asserting that had dogs instead of rabbits been employed for the present work years instead of months would have been required to obtain an understanding of the simpler principles ruling biliary lesions—principles that are of evident general application, and to be derived as surely from observations upon one mammalian species as another.

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[5] Quincke, H., and Hoppe-Seyler, G., in Nothnagel, H., Specielle Pathologie und Therapie, Vienna, 1899, xviii, 59.

[6] Harley, V., and Barratt, W., *J. Path. and Bacteriol.*, 1901, vii, 203.

Method.

In work already reported,[7] we have taken advantage of the singular fitness of rabbits for experiments upon the liver which in this species consists of two well separated masses, each with its own bile duct and blood vessels. The smaller, or lobe mass, as it may be called, forming approximately one-quarter of the whole —23.7 per cent after ablation of the caudate lobe (4 per cent)—will accommodate on emergency the entire portal stream and maintain the animal in good health when the larger mass, or main liver (72.3 per cent), is ablated. The liver of the rabbit adapts itself to new conditions far more rapidly than that of the dog or cat. Thus, for example, the gradual parenchymal atrophy and hypertrophy which follow local portal diversion require but 2 months in the rabbit for their approximate completion, whereas in the dog several times this period is required.[7] There exists, however, a very considerable drawback to the clinical study of jaundice in the rabbit in the fact that the bile pigment of the animal reacts but poorly to the ordinary color tests. Our work has not been of a sort to make this difficulty felt. A second and serious potential objection to rabbits is to be found in the intercurrent hepatic cirrhosis prevalent in certain localities. The lesion has been rare in our experience as in that of some others,[8] and individuals showing it at operation, the first step in every experiment, have been discarded. As a further control to the condition of the liver, the caudate lobe has regularly been ablated for section. The total bulk of the organ is thus reduced by about 4 per cent, as already stated.

The rabbits weighed from 1,400 to 2,500 gm. The general method of operation has been described in another connection.[7] Bile ducts to be ligated were dissected free of the surrounding structures and tied twice with fine black silk, but not cut, since this precaution against a restoration of continuity was found unnecessary. The ducts are extremely delicate. Almost all the postoperative mortality was due to rupture of them above the ligature. When a branch of the portal vein was to be tied, it was dissected out. Special care was always taken to avoid injury to the hepatic artery, for this in the rabbit usually results in more or less widespread necrosis. Recovery from operation was in general uneventful. For the better analysis of the lesions most of the animals were killed early, with chloroform or by bleeding from the heart. Bits of the liver tissue were placed upon agar and in bouillon, and additional stroke and loop inoculations were made. Infection was rare, and instances showing it were ruled from consideration. A scattered, localized coccidiosis was fairly frequent, however. It had no evident complicating effect upon the liver changes.

Zenker's fluid, formaldehyde, and alcohol were used as fixatives.

[7] Rous, P., and Larimore, L. D., *J. Exp. Med.*, 1920, xxxi, 609. All operations were performed under ether anesthesia.

[8] Grover, A. L., *J. Am. Med. Assn.*, 1915, lxiv, 1487.

The amount of the bile and the pressure developing within obstructed ducts are both relatively great in well fed animals.[9] To this circumstance and to differences in the character of the food[10] are probably attributable most of the considerable quantitative variations that may be observed in lesions resulting from a single procedure. Our animals were not fed for 18 hours prior to operation, but immediately thereafter they were placed upon the mixed diet of normal rabbits.

Results of Total Obstruction.

Nearly all attempts to obtain by experiment an understanding of biliary lesions, from Leyden's time on, have centered about the production of total stasis by ligation of the common duct; and there exist many descriptions of the ensuing changes.[11] Our observations have but confirmed the general findings, while yielding an interpretation of them that has proven productive.

The rabbit secretes bile so copiously—136.8 gm. per kilo of animal in 24 hours, according to Heidenhain[10]—that obstruction of the common duct is followed practically at once by marked distension of the passages. Within a few hours bile-stained parenchymal necroses appear, of all sizes, from those that involve but a single cell to wedge-shaped areas, the so called biliary infarcts, 0.5 cm. or more in diameter. The larger ones are stained a bright yellow-green and frequently show hemorrhages. All are obviously the result of direct extravasation of the bile, either within the lobule or at its periphery where the smallest collecting ducts have ruptured. An identical rupture at this latter point, the weakest one in the collecting system, occurs on the injection under pressure of fluid into the common duct.

Within a few days dilatation of the larger ducts becomes great, and pericholangitis develops, followed soon by a spreading, stellate cirrhosis. The changes are most marked in the region of the larger portal spaces, but there is active proliferation of the connective tissue throughout Glisson's capsule, and every lobule is soon enclosed and encroached upon peripherally. The smaller necroses are replaced by proliferation of the neighboring liver cells, but with the larger

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[9] Bürker, K., Arch. ges. Physiol., 1900–01, lxxxiii, 241.

[10] Heidenhain, R., in Hermann, L., Handbuch der Physiologie, Leipsic, 1883, v, pt. 1, 256. Hooper, C. W., and Whipple, G. H., Am. J. Physiol., 1916, xl, 332.

[11] Richardson has an excellent brief description (Richardson, M. L., J. Exp. Med., 1911, xiv, 401). For one more extensive, with a survey of the literature, see Fiessinger, N., and Roudowska, L., Arch. méd. exp. et anat. path., 1914–15, xxvi, 18.

this is impossible, owing to involvement of all the lobular elements, and organization takes place by a connective tissue invasion. The surviving parenchyma becomes more and more jaundiced, and numerous intralobular bile thrombi appear. Small scattered necroses continue to develop, but no large ones form after the first days. The lobules become very irregular and diminish in size through the encroachment of connective tissue, and the latter rapidly penetrates in thin strands between the cell cords to the central vein. Within 3 weeks the liver has become a roughly hobnailed organ, with ducts everywhere greatly distended, and a cirrhosis that is at once periportal, or, more properly speaking, pericholangitic, unilobular, irregularly intralobular where necroses have been replaced, and almost regularly intralobular here and there where the connective tissue penetration is diffuse. Throughout the older cirrhotic tissue are great numbers of new-formed bile ducts. Despite the extensive parenchymal destruction, the liver remains of about the normal size. Thrombosis of portal branches not infrequently occurs, with some resultant atrophy of liver parenchyma, while elsewhere an hypertrophy may be observed when the condition of the animal permits, as is rarely the case. Always there is great emaciation, and death usually ensues within 4 or 5 weeks. Jaundice appears after the first few days of obstruction.

No human lesion resembles this one more than remotely, and attempts to utilize it in explanation of the biliary cirrhoses of man have been almost given up. Yet when its histologic components are considered separately the fact becomes plain that certain of them find their replicas in one kind of human liver cirrhosis, and certain in another. In a word, the rabbit lesion is mixed, involving injury from stasis throughout the entire length of the biliary tract. By altering the conditions it is possible, as we shall show, to localize the injury of stasis to regions corresponding to ducts of a single level or order, and thus to obtain cirrhoses of uncomplicated type which closely resemble the biliary cirrhoses of man.

Results of Local Obstruction.

The results of tying off the bile duct to the main liver mass of the rabbit, some three-fourths of the entire organ, while leaving that to the lobe mass free have been briefly described by Nasse.[12] We have repeated his experiments and can confirm his statements as regards the early changes, these alone having importance for the present work.

[12] Nasse, *Verhandl. deutsch. Ges. Chir.*, 1894, xxiii, pt. 2, 525.

The effects of the sudden stasis on the main liver, though outspoken, are far less marked than after total obstruction. Dilatation of the bile ducts and gall bladder is relatively moderate, biliary necroses are small and appear only during the first few days, and the animal remains in good condition, never becoming jaundiced. The occluded parenchyma shows no bile tinting after the 1st week. Cirrhosis makes its appearance gradually and differs from that of total stasis in its slow, orderly progress, almost complete failure to invade the lobuli, and, in the subordination of stellate growth about the larger ducts, to a diffuse interlobular proliferation (Fig. 1). But new-formed bile ducts are still a prominent feature. The parenchyma undergoes a rapid atrophy. Within a few weeks the mass is much smaller, diffusely cirrhotic, and relatively bloodless; and by the end of 4 months it is reduced to a tag from which parenchyma and new-formed bile ducts have alike disappeared.[12] There is only the slightest hobnailing at any time. As atrophy of the main liver progresses the lobe mass undergoes hypertrophy and eventually reaches the size of the entire normal organ.

The cirrhosis here approaches the pure unilobular in type, for penetration between the cell cords is almost wholly absent (Fig. 1). But there still exists the complication of a stellate periportal proliferation, with notable dilatation and pericholangitis of the larger ducts. Nasse attributed the atrophy to closure of the secretory channels of the liver, likening the result to that of ureteral ligation. This though, is no sufficient explanation, since under the conditions of total obstruction the liver holds its size, and there may even be some local hypertrophy despite the progressive general emaciation. With local obstruction similar attempts at repair are never seen, although the animal stays in good condition.

The Factor of Portal Obstruction.

It has seemed to us probable that the mildness of the biliary lesions, including the cirrhosis, after local obstruction, and the parenchymal atrophy as well, are due in great part to a single factor, namely, partial deflection of the portal stream from the affected region. Several facts support the view. When the duct from the main liver is tied the lobe mass becomes engorged with blood and hypertrophies just as when its portal blood supply is increased by obstructing the venous trunk to the main liver;[7] while a sufficient cause for obstruction is to be found in the dilatation under pressure of the main bile

channels of the liver.   Betz showed in 1862[13] that a very moderate increase of pressure within the ducts, far less than that developing after total obstruction,[14] can greatly hamper the portal flow.   That it does hamper it in the present case, and early, was indicated in one of our animals examined 5 days after ligation of the bile duct to the main liver.   The lobe mass was already much engorged and enlarged, although as yet no connective tissue changes had occurred in the main liver that could be invoked to explain a venous obstruction there. The effect on hepatic tissue of the occlusion of portal branches has already been studied by us.   There ensues an orderly atrophy with lessening of the biliary activity, and ultimately all parenchyma disappears.   Precisely such an atrophy, though one more gradual, results from local biliary stasis; while a diminished biliary activity, at least as regards secretion into the ducts, is indicated by the moderate dilatation of the latter in comparison with their condition when stasis is total, and by the relative rarity of biliary necroses and the slow course of the connective tissue changes.

To obtain additional evidence we have studied the effects of obstructing simultaneously the bile duct and portal branch of a liver mass.

Experiment 1.—Two 1,500 gm. rabbits were operated upon under ether and the bile duct and portal trunk to the main liver twice ligated, just above the caudate lobe.   The latter was not taken out.   The livers appeared normal at operation, and subsequent examination of the hypertrophic lobe mass showed that cirrhosis had been absent.   The animals were killed by bleeding from the heart 5 days and 21 days respectively after operation.   They had been in good condition and unjaundiced.

In three other rabbits a mere ligation of the bile ducts to the lobe mass was followed by an occluding thrombosis of the accompanying portal branch.   The occlusion must have developed soon after operation, for when the animals were killed the histological findings were identical with those observed in the main liver after simultaneous ligation of both duct and vein.

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[13] Betz, W., *Sitzungsber. k. Akad. Wissensch. Math.-naturw. Cl., Wien.*, 1862, xlvi, 238.

[14] Herring, P. T., and Simpson, S., *Proc. Roy. Soc. London, Series B*, 1907, lxxix, 517.

Experimental Unilobular Cirrhosis.

Different stages of the same lesion were found in these rabbits. There was little dilatation of the large bile ducts and only a slight, transient jaundice of the tissue in stasis. A few small parenchymal necroses were seen early, but none later. The lobules rapidly diminished in size by simple atrophy, yet cirrhosis appeared more slowly than when the bile duct alone was obstructed, and, while ringing all the lobules about with a tissue containing many little, new-formed ducts, had not the least invasive tendency. It was, in sum, a pure, evenly distributed, monolobular cirrhosis. Stellate proliferation about the larger ducts was practically absent, as might have been expected from the absence of any marked stretching of their walls. With this exception the picture so entirely resembled that found after occlusion of a bile duct only (Figs. 1 and 2) as to constitute strong evidence for the view that obstruction of the portal stream has much to do with the lesions occurring under such circumstances.

Further Influence of the Portal Obstruction.

The deflection of the portal stream from a liver region in which the bile ducts are dilated as the result of obstruction is probably only partial at first. Later, when cirrhosis develops, it becomes much more nearly complete, as shown by the relatively bloodless condition of the tissue at a period when much parenchyma is still present. Perhaps the arterial circulation is now compromised. Certainly a portion of the blood that comes to the liver passes on to the vena cava through capillaries in the cirrhotic tissue[15] and thus fails of contact with the parenchyma. Pigment secretion into the ducts completely ceases; their dilatation, while still very moderate, comes to a standstill; and the fluid in them becomes colorless and watery. Yet, as our previous work has shown, a main liver mass long deprived of all portal blood, and in advanced atrophy as result, is still capable of forming bile in quantity.[7] The pressure, though, against which secretion takes place into the ducts is greatly diminished, as the following experiment proves.

[15] Ackermann, *Virchows Arch. path. Anat.*, 1880, lxxx, 396.

Experiment 2.—The portal trunk to the main liver mass was ligated in two rabbits of approximately 1,800 gm. weight, and the caudate lobe ablated as usual. 17 and 23 days later, respectively, the bile duct from the mass was ligated just below the entrance of the cystic duct, and 2 and 3 days later the animals were killed and immediately examined. The main liver had atrophied in both instances to less than one-third of the normal weight, while the posterior lobe mass had undergone a corresponding hypertrophy.

In a number of other rabbits the operative procedures were the same, but a gall bladder fistula was made in addition. Certain of the instances in which bile collection was successful have already been described.[7] Sometimes occlusion of the fistula developed, and the animals were killed at various periods thereafter. The findings in such cases deserve consideration with those of Experiment 2 proper, since the conditions are essentially similar.

In these animals with an advanced atrophy of the main liver as result of portal ligation, not the least icteric tinting of the parenchyma or dilatation of the bile passages and gall bladder followed occlusion of the duct; and the passages contained merely a little watery, colorless fluid. Obviously a very slight increase of pressure within the ducts had been sufficient to check secretion into the normal channels. It follows that as hepatic atrophy progresses after local biliary obstruction the chance that lesions will occur through rupture of the ducts, or stasis within them, becomes negligible. Yet when the material for bile formation is provided in unusual quantity a parenchyma deprived of the portal stream can secrete bile into the ligated duct against a considerable pressure, as will now be illustrated by an experiment done with another end in view.

Experiment 3.—A hemolytic and hemagglutinative serum of high titer was obtained by repeatedly injecting a goat with washed, rabbit red cells. A small amount of the serum when given intravenously to rabbits was found to bring about blood destruction over a period of many hours without demonstrable liver injury, whereas large quantities caused necroses. With a properly regulated dosage there was no escape of hemoglobin by way of the urine, even when more than half of the blood of the animal was broken down in the course of only 2 days.

The portal vein and bile duct of the main liver of a 1,700 gm. rabbit were ligated just below the entrance of the cystic duct. 4 days later, when the animal had recovered from the operation and was in good condition, intravenous injections were begun of small doses of the anti-rabbit serum, followed at short intervals by the transfusion of washed, compatible, rabbit red cells suspended in a little salt solution. The amount of serum given was always well below that causing liver changes, but it effected great blood destruction, necessitating five large transfu-

sions to maintain the hemoglobin at the original level. In a period of 11 days 73 cc. of sedimented corpuscles, an amount equivalent to the cell content of 162 cc. of the blood of the animal, or nearly twice the normal total, had been destroyed as the result of but four injections of serum, or 1.7 cc. in all. The animal now possessed a hemoglobin of 86.5 per cent (Palmer) as compared with 82.6 per cent prior to the experiment. It was killed and autopsied. There was at no time any jaundice.

Three rabbits were submitted to a similar experiment for a shorter period of time, and in two others repeated injections were made subcutaneously of large amounts of hemoglobin prepared by the method of Minot and Sellards. No jaundice was observed in any animal.

In these rabbits the bile passages of the main liver and the gall bladder as well were greatly distended and with an abnormally thick, dark green bile. The liver tissue was deeply jaundiced. The changes cannot be contrasted with those in the animals of Experiment 2, since atrophy of the main liver was advanced in the latter before the bile duct was obstructed. They should be compared with the results in Experiment 1, in which a bile duct and portal branch were simultaneously obstructed. Not only was the local accumulation of bile far more copious and the distension of the ducts greater than in the few instances of this experiment, but both were much more pronounced than in any of the rabbits in which, following Nasse's example, we ligated only the duct of the main liver, leaving the portal circulation untouched. It follows that the failure of a liver region deprived of the portal stream to secrete bile against pressure cannot be laid to an essential disability of the cells. In a previous paper evidence has been brought forward that the cells are handicapped in competition with the parenchyma receiving all the portal blood.[7]

Experimental Intralobular Cirrhosis.

A stellate cirrhosis about the larger bile ducts is not rare in human beings as the result of obstruction, with or without infection, and has frequently been brought about experimentally in animals. We have deemed its production in rabbits, apart from other changes, as unnecessary, although logically considered this would be a further step toward separating out the components of the mixed lesion that follows occlusion of the common duct. To obtain, on the other hand,

a type of stasis, and thus presumably of cirrhosis, localized within the lobules, seemed highly desirable. *A priori*, one would expect to meet many difficulties, since it is known that a back pressure throughout the duct system which suffices to cause partial stasis in the lobules almost invariably results in a rupture of some of the bile radicles within Glisson's capsule, and always in connective tissue proliferation about the larger ducts. Our success in obtaining a predominantly intra-lobular stasis has been the outcome of varied attempts. The condi-tion, and its corollary, a diffuse intralobular cirrhosis, can regularly be brought about in the lobe mass of the liver by diverting the whole portal stream to the latter and ligating the efferent duct. A like re-sult is not produced, we find, when the larger mass of the main liver is subjected to the same procedure, an experiment already performed by Steenhuis.[16]   The cirrhosis that then occurs is predominantly extralobular.

Experiment 4.—In eighteen rabbits weighing from 1,500 to 2,400 gm. the bile duct of the posterior lobe—or ducts, for there may be as many as three—was ligated, the portal trunk to the main liver tied off just above the branch to the caudate lobe, and this last ablated as usual. The animals remained in good con-dition and unjaundiced. They were killed after intervals of from 1 to 33 days.

The operation as described is frequently followed by complications; and the findings in many animals not included in the above number have been ruled from consideration on this account.

The course of the changes has been carefully followed. There is an initial turgor and hypertrophy of the lobe mass resembling that after ablation of the main liver,[17] or simple ligation of the portal branch of the latter,[7] and due of course to the same marked, local increase in the portal stream. Superimposed are destructive changes referable to the bile stasis. The stasis, though brought about by a ligature on the principal duct of the lobe, causes remarkably little dilatation of the latter and still less of its large branches within Glisson's capsule. These are never abnormally prominent on the cut surface of the liver, though mi-croscopically a slight distension and thickening of their walls may be observed after a time, with occasionally some pericholangitis. In the course of several weeks the main duct usually attains a diameter of about 3 mm., that is, becomes twice the normal size, and contains stasis bile, a watery fluid with a few green

[16] Steenhuis, T. S., Experimenteel en Kritisch Onderzoek over de Gevolgen van Poortaderafsluitung, Thesis, Gröningen, 1911.

[17] Ponfick, E., *Virchows Arch. path. Anat.*, 1889, cxviii, 209; 1890, cxix, 193; 1895, cxxxviii, suppl., 81.

solid particles. The brunt of the obstruction falls on the smallest bile radicles in Glisson's capsule, and still more on the canaliculi within the lobules themselves, as shown by the bile thrombi here found and the numerous, minute parenchymal necroses. Such necroses appear within a few hours after the ligation and new ones develop day by day. Usually they cannot be seen with the naked eye, involving, as they most often do, one to three or four cells scattered throughout the lobule from periphery to center. The dead cells stand forth prominently in the liver cords, staining a bright pink with eosin, and lacking nuclei (Fig. 3). By the 3rd day they are numerous, especially in the outer and mid-zone of the lobule, and occasionally a few larger necroses involving ten or fifteen cells may now be present. Some of the latter are undoubtedly the *Netz-Nekroses* which Steenhuis[16] saw in liver tissue hypertrophic as result of an increased portal flow, but others have the character of frank biliary necroses. Very exceptionally they involve considerable lobular segments and may then attract the unaided eye. In animals showing them the smaller, disseminated necroses are especially numerous. It is the latter that characterize the lesion and continue steadily to be formed. Being purely parenchymal they are rapidly replaced by proliferation of the lobular cords without connective tissue participation, and their number is never sufficient at any one time to render them confluent. They appear to have no relation to the small, scattered, intralobular bile thrombi that, during the first few days, and then only, are numerous enough to attract attention.

The severity of the lesion varies largely with the individual. By the 3rd day in some cases, or the 6th or 7th in others, a new element makes its appearance; namely, a proliferation in the finer ramifications of Glisson's capsule. Fibroblasts begin to penetrate within the enlarging lobules (Fig. 4), and by the 12th day as a rule many thin strands of them can be found, so far toward the central vein and so isolated in cross-section that were the absence of connective tissue within the normal parenchyma not well attested[18] one would conclude that they had developed *in situ*. New-formed bile ducts have now made their appearance in the interlobular connective tissue which is definitely increased in amount.

The lobe mass at this time is highly interesting in the gross. It is enlarged, sometimes to double its normal size, with rounded contours and blunt edges. The bulging hypertrophic lobules render the surface slightly and regularly uneven. The tissue is firm and somewhat inelastic, and the knife meets a smooth, non-crepitant resistance. The color is gray-pink, in contrast to the purple of the main liver. When the vessels are severed nearly all the blood is forced out by the turgid tissue, and the general tint is then a uniform, pale buff, or putty color. On the surface laid open the lobules can with difficulty be made out, slightly raised, in a matrix of gray, translucent, connective tissue which appears to penetrate and overgrow them. Sometimes their position can be made out only through the situation of the gaping central veins. There is now no definite jaundice of the tissue, though its yellowish tint is suggestive. Earlier, during the

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[18] Mall, F. P., *Am. J. Anat.*, 1906, v, 227.

first few days after the ligation, the enlarged lobe may be a greenish red-purple, though jaundice of the animal never occurs.

By the 21st day the lobe mass, still in process of hypertrophy, contains nearly as much connective tissue as parenchyma. Almost everywhere fibroblasts have penetrated to the central veins of the lobules, though so regularly that the original pattern of the liver is still fairly maintained. The cell cords, though, are distorted and much fewer. The necrosis of individual cells and small cell groups is still going on, as is also proliferation of the surviving parenchymal elements which individually may be much above normal size. The interlobular connective tissue is greatly increased, both relatively and actually, and everywhere throughout it are new-formed bile ducts. About some of the small hepatic veins is a collar of young connective tissue containing in its midst irregular strands of small, compressed looking cells, superficially suggestive of new-formed bile ducts but in situation widely separate from the latter and with tinctorial differences that identify them as parenchymal.

At this time the lobe mass is of a pale flesh color and appears almost bloodless compared with the main liver (Fig. 9). In truth it is so, owing to interference with the portal stream by the cirrhotic tissue, which usually begins before the 12th day and has now become marked. Venous collaterals open, sometimes as a *caput Medusæ*, when the omentum is attached by adhesions to the old wound, more usually as small, direct anastomoses between the portal branches and vena cava, and oftenest as dilated Charpy's veins along the gastrohepatic omentum to the main liver. But all of the new channels fail as a rule to avert a serious chronic passive congestion. As result the spleen is changed into a tense cylinder (Fig. 9) and may weigh more than 4 gm., nearly six times the average for normal rabbits of similar size. Occasionally ascites occurs. On cutting into the lobe mass its large, portal channels are found to end bluntly in small veins.

The surface of the lobe is still finely rugose, and its substance is now extremely resistant to the knife, though non-crepitant. When laid open the glistening, bloodless, pinkish gray tissue shows no distinct pattern of lobules, but these can usually be made out when viewed obliquely, since they are slightly raised above the general surface. Some appear larger than normal whereas others blend with the connective tissue. The main liver mass fails to undergo the atrophy which in the lack of a functional need would be its fate.[7]

By the 30th day cirrhosis has progressed much further and so has the development of collaterals which shunt the portal blood around the lobe mass. The adequacy of the new channels is now shown by the disappearance of all signs of passive congestion. The lobe mass has in consequence come to lie outside of the venous stream, being reduced to much the same circulatory condition as if its portal branch had recently been tied. Hypertrophy ceases, and atrophy takes its place. But though the lobe mass is still much above normal size little parenchyma is left to undergo retrogression. The mass consists almost entirely of connective tissue with an abundance of new-formed bile ducts and some round cells. Here

and there are to be found small islands of liver cords, and separated cords and cells (Fig. 5), some of them dying, though whether as a result of the bile stasis or of the cirrhosis we have not sought to discover. The liver destruction has far surpassed that compatible with life were the whole organ affected. Beyond the 33rd day the changes have not been traced.

Altogether the type of cirrhosis is as purely intralobular as is compatible with the fact that the lobules normally contain no connective tissue,[18] so that its appearance and proliferation within them must of necessity take place by penetration from without.

Influence of an Increased Portal Stream.

The secretory activities of a lobe mass receiving the entire portal stream but with bile duct ligated, as in the preceding experiment, become strikingly evident when the duct from the main liver is also tied with result in total obstruction. There is now no relief for the lobe mass such as might be afforded through the efforts of a parenchyma with unobstructed bile outlet.

Experiment 5.—In ten rabbits of 1,500 to 2,200 gm. an operation similar to that of Experiment 4 was successfully performed and in addition the bile duct from the main liver mass was ligated. In eight cases the gall bladder was removed in order to render the conditions in the main liver more strictly comparable with those in the lobe mass. The animals died, or were killed, after periods of 1 to 12 days. Cultures taken from such as died showed that the liver lesions were not referable to infection; and as further proof the histological findings agreed in all ways with those of animals killed while still in good condition.

The animals of this experiment were more seriously affected than if total biliary stasis had been produced in the ordinary way by obstruction of the common duct. A sufficient explanation is to be found in the radical circulatory derangement.

Only two animals survived for as long as 12 days. Jaundice always developed within 48 hours, and rapid emaciation was the rule; yet despite the poor general condition the lobe mass of the liver underwent some hypertrophy. Its turgid tissue became brightly and evenly bile-stained—green, or ocher-colored. The surface remained smooth, and at no time were biliary necroses perceptible in the gross (Fig. 10). The lobules were definitely enlarged and appeared regular, save that near the central vein there was some opacity with a brilliant bile tinting. The impression here gained was not that of a diffuse necrosis but of intense im-

pregnation of living tissue with bile pigment. This was the actual state of affairs, as sections showed. Toward the center of the lobules pigment existed in great quantity as small bile thrombi (Eppinger), as large, rounded, brownish black masses (Fig. 6), intracellular particles, and as a diffuse stain for groups of dead parenchymal cells. No such marked condition has to our knowledge been encountered heretofore, though for that matter the factors in its production can scarcely be expected to come together naturally.

The earliest histological changes were like those observed in the companion Experiment 4, in which the duct of the main liver was left open. There was the same scattered necrosis of individual cells throughout the lobule, but in much more pronounced form (Fig. 7), and dilatation of the ducts was greater. Small concretion-like pigment "thrombi" appeared early here and there between the parenchymal cells, and after but a few days the pigmentary accretion was, as already described, enormous (Fig. 6). The number of dead cells grew rapidly, and close to the center of the lobules larger biliary necroses occurred, some deeply impregnated with bile, but none of such size as to attract the unaided eye. These features acted to modify the rapidly developing cirrhosis, which in the animals surviving longest was intralobular both by diffuse invasion and by the replacement of necroses. Pericholangitis of the larger ducts was absent. At the 12th day active division of the surviving parenchymal cells was still going on; some elements were of greatly increased size, and nuclear irregularities were frequent.

The main liver showed only the lesions that ordinarily follow obstruction of the common duct, combined with the slight atrophy inevitable to deprivation of the portal stream. The local accumulation of pigment was slight, as compared with that in the posterior lobe mass, yet large biliary infarcts were numerous (Fig. 8).

The extraordinary impregnation with pigment of the posterior lobe mass (Figs. 6 and 7) contrasts strikingly with the slight jaundice of the main liver (Figs. 8 and 10). Since biliary activity is dependent, in large part at least, upon portal flow, and this is greatly different in the two liver portions, such a contrast need not occasion surprise. The absence of gross necroses, though, in the posterior lobe mass is difficult to reconcile with the pronounced biliary leakage there occurring save on the assumption that the increased portal flow provided a circulation to cells that otherwise would have been deprived of it by the extravasations.

The Factor of Safety in Bile Elimination.

The tissue of the lobe mass in Experiment 4 was jaundiced only during the first few days after operation. Experiment 5 clearly shows that bile pigment must have been prevented from accumulating here,

as well as in the organism generally, through the activities of the main liver. When there exists only an uncomplicated biliary obstruction of the main liver, the posterior lobe mass functions in the same way to prevent a general jaundice, as has already been noted. It is remarkable that the literature contains no direct recognition of the fact that a small portion of the liver may serve for the whole as far as regards the elimination of bile pigment. On the contrary, one finds everywhere the statement that the severity of jaundice is directly proportional to the amount of liver tissue in stasis; and small, local lesions have been invoked to explain its clinical occurrence. The subject merits a separate paper and will be considered here only in so far as it affects our theme. But this it does to a considerable degree.

The factor of safety in bile elimination is strikingly illustrated by some recent experiments for another purpose performed by one of us and Dr. Philip D. McMaster. The bile duct from the main liver was tied, and so too was the portal branch to the lobe mass from which the caudate lobe was ablated as usual. Owing to the increased portal stream to the main liver the tissue of the latter must be supposed to have formed bile in unusual quantity, and this found no outlet by the normal route. Yet the eight rabbits of the series remained in excellent condition and unjaundiced, although the bile could be eliminated only through the lobe mass, less than a quarter of the total substance of the liver; and supplied with blood only through the hepatic artery, which, judging from observations on dogs,[19] provided only about 40 per cent of the normal blood supply.

Local Effects of Increased Bile Secretion.

It has repeatedly been suggested on the basis of clinical evidence that increased secretion, or the secretion of an abnormally thick bile, may lead to stasis within the ducts and thus injure the liver eventually. Hanot's cirrhosis was at one time attributed to such cause. But certainly small portions of the liver of the rabbit can rid the body of large amounts of the animal's highly irritant bile without suffering injury. This was true in all of the instances of compensatory secre-

[19] Macleod, J. J. R., and Pearce, R. G., *Am. J. Physiol.*, 1914, xxxv, 87.

tion described in the present paper. Even when the bile is thickened as the result of hemolysis and the whole brunt of its elimination is abruptly thrown upon the lobe mass of the liver, the latter suffers no damage visible histologically. Experiment 3 was originally devised to test this point.

<div align="center">DISCUSSION.</div>

<div align="center">*Physiological Pathology of the Lesions.*</div>

Rabbit bile that has escaped from the ducts is highly injurious to the tissues, causing necrosis even of the liver parenchyma.[20] In this fact and in the differing paths by which the fluid may leave the collecting tract under various conditions of obstruction are to be found primary reasons for the diverse types of biliary cirrhosis which we have produced experimentally.

The cirrhosis which follows ligation of the common duct is, as has already been pointed out, a mixed lesion. So considerable is bile secretion despite the stasis, that no part of the collecting tract from the intralobular canaliculi to the main duct, inclusive, escapes the effects of the irritant fluid. Pressure changes also occur, and the local condition is probably aggravated by the resecretion of biliary constituents from the heavily charged blood,[21] which would greatly favor accumulation within the hepatic tissue. Though most of the secretion must continue to find its way back into the blood and lymph, the process cannot but be faulty when the body fluids are already heavily laden.

The progressive atrophy and orderly cirrhosis that result from obstruction of a single large bile duct are traceable to the interaction of two opposing sets of factors, one set vicious, the other tending to relieve the conditions. The initial bile stasis leads to rupture of some of the finer ducts with parenchymal necrosis, and its continuance to an interlobular and pericholangitic connective tissue growth. Probably this cirrhosis interferes eventually with the general blood

[20] Bunting, C. H., and Brown, W. H., *J. Exp. Med.*, 1911, xiv, 445.

[21] Bile constituents injected into the circulation are rapidly taken out by the normal liver—*vide* Stadelmann, E., Der Icterus, und seine verschiedenen Formen, Stuttgart, 1891.

supply. There is no doubt that the portal flow is partially diverted from the beginning through the encroachment of distended bile ducts on the stream bed, and hence there ensues a gradual atrophy of the parenchyma such as follows any local portal deprivation.[7] But while destruction is thus being effected through a combination of several influences others act to limit the injury. The liver portion with duct still open keeps the organism more or less free from bile and thus tends to prevent resecretion into the region of stasis; secretion is still further reduced by the diverted portal flow;[7] and the direct effects of such stasis as nevertheless ensues are lessened by the passage of biliary constituents from the obstructed tissue into a blood and lymph relatively free from them. As parenchymal atrophy advances the pressure under which bile is secreted into the ducts decreases greatly (Experiment 2), and in consequence their rupture, with extravasation, no longer occurs. Furthermore, with the passing of time the functional responsibilities of the tissue in stasis decrease, owing to hypertrophy of the unobstructed liver mass. And so through several means the local changes are rendered mild and orderly. Similar lesions from similar causes, but with less of cirrhosis and a more rapid atrophy, as would naturally follow from the circumstances of the case, are seen when a bile duct and its corresponding portal branch are occluded at the same time (Experiment 1).

The predominantly monolobular cirrhosis which follows the occlusion of a single duct, and the purely monolobular lesion that develops when the corresponding portal branch is also tied, are referable not to rupture of the ducts, which is negligible, but to continued passage through their walls of the irritant bile. When a colored solution is introduced into the bile passages under a pressure that fails to rupture them it escapes with ease through the walls of the finest radicles in Glisson's capsule.[18] And precisely here, as indicated by the connective tissue lesion, does the bile pass into the tissues under the circumstances we are now discussing. This path of egress would seem sufficient to relieve the stasis when secretion has been cut down by the ligation of the portal branch to the affected region, for under such circumstances lesions are not observed elsewhere in the liver. When the duct alone has been ligated secretion and stasis are both greater, and in addition to the monolobular cirrhosis a stellate proliferation is then found about the large, distended ducts.

No clear reason is evident for the intralobular situation of the stasis that results from obstructing a single bile duct and greatly increasing the local portal flow. The changes that ensue, though, are easily understood. An increased portal flow of itself fails to induce connective tissue changes.[17] They may be attributed in the present instance to the numerous bile leaks within the lobules, leaks which also cause a profusion of punctate necroses. The lymph from the affected tissue comes away more or less laden with bile. It passes, not direct from the lobules into the formed lymphatics of Glisson's capsule, but first through unlined spaces between the connective tissue cells.[18] Here its biliary component has abundant opportunity to cause irritation, and here consequently at the edge of the lobules connective tissue proliferation occurs, and fibroblasts penetrate rapidly between the liver cords, following up, so to speak, the path of the irritant fluid. A diffusely intralobular cirrhosis is the result. The absence of large biliary infarcts, despite the great secretory activity of the tissue (Experiment 5), may be due in part to the support given the walls of the bile radicles by the increased portal pressure, which would also act to maintain the blood current despite local extravasations so that the latter affect relatively few cells. The observation has been made (Experiment 5) that when there is an increased portal flow in one part of the liver, an absence of such flow in the remainder, and biliary obstruction in both, gross biliary necroses fail to occur in the first mentioned liver portion, although the jaundice there is extreme, whereas in the second portion, which is unjaundiced, and must be relatively inactive, large infarct-like necroses appear (Fig. 8). To judge from this there would appear to be much in the current belief that biliary necroses, though caused in some part by the direct injury of cells by extravasated bile, owe their size when large to interruption of the blood current.

Interpretation of Human Lesions.

Many facts warrant the application of the principles here set forth to the frank biliary lesions of human beings. The essential likeness of the changes in man and the rabbit has been briefly indicated in an earlier part of this paper. As Quincke and Hoppe-Seyler put the

matter, summing up their more extensive comparison: "From all this it would seem that the findings in guinea pigs and rabbits are analogous in many ways to those in human beings."[6] But it is in essentials only that the findings are analogous. The relatively innocuous character of human bile, the slow rate at which it is secreted, the greater frequency of infection in areas of stasis, all act to vary the picture. Furthermore, the liver parenchyma may, like that of the dog, respond rather slowly to changed circulatory conditions[7] such as bile stasis induces. Certainly the intralobular bile canaliculi are, like those of the dog and cat, relatively inaccessible to pressure exerted through the large ducts,[18] so that they are only slightly implicated by stasis in these latter.

The forms of cirrhosis associated with Hanot's name cannot on present evidence be classed as of frank biliary origin. Nevertheless, our observations bring something of clarity into a consideration of them. Whether there exists an entity of the sort described by Hanot has been much debated, and there is great discordance in its definition by recognized authorities. No one disputes, on the other hand, the occurrence in man of a peculiar, progressive, hepatic cirrhosis, accompanied by more or less jaundice, and resulting usually in an enlarged liver in which are found inflammatory lesions of the smaller bile ducts with signs of stasis in them. It is our belief that the diversity of the liver changes is referable to differences in the duct levels at which the injurious agent is active, differences which in the rabbit lead, as we have shown, to cirrhoses of strikingly individual character. In rabbits, and with bile as the irritant, a localization to the intralobular bile canaliculi leads to a diffuse intralobular cirrhosis, whereas when the smallest bile radicles in Glisson's capsule are affected there results a pure monolobular cirrhosis, and implication of the large bile channels leads to a stellate proliferation about them. Parallel instances, pure and mixed, of all except the last mentioned lesion, are to be found within the group of the hypertrophic biliary cirrhoses of man. The rapidly progressive type of the disease, frequent in Indian children, is almost entirely of intralobular character, as Gibbons' excellent description shows.[22] There

[22] Gibbons, J. B., *Sc. Mem. Med. Off. India*, 1891, vi, 51.

is here a marked degeneration of the liver cells.   According to Kaufmann,[23] with whom relatively few agree, intralobular growth is a distinctive character of Hanot's disease.   In Lereboullet's monograph[24] most of the instances are monolobular, with occasionally an intralobular and sometimes a perilobular involvement.

The hypertrophy of the surviving parenchyma in Hanot's cirrhosis is not more difficult to understand than that usual to atrophic cirrhosis and here regenerative in character.[25]   In explanation of the continued connective tissue growth which renders the liver large, one need only invoke the constant stimulus to proliferation that is undoubtedly present, as the angiocholitic lesions show.   By contrast, injury is intermittent in the atrophic cirrhosis of Laennec, and at death, when a contracted scarring is found, often no signs of recent damage can be made out.   The round cell aggregations present in hypertrophic biliary cirrhosis need no other cause than the chronicity of the inflammation.   And the not infrequent chronic passive congestion occurring at a late stage with or without a marked diminution of the size of the liver, is referable to secondary connective tissue shrinkage and to the localization of the cirrhosis in the individual case.   In our rabbits with hypertrophic biliary cirrhosis chronic passive congestion regularly developed as a late feature.

The ability of the least harmful bile of which the effects have been carefully followed, that of the cat, to elicit eventually a liver cirrhosis in the absence of infection[6] might well lead one to stress this factor as a cause of connective tissue changes in the human liver.   The occurrence of intense local bile-staining without such lesions, a phenomenon not infrequent at autopsy in liver diseases, carries no weight in this connection, since the chronicity of the local stasis is here unattested.   But Hanot's cirrhosis has certainly a varied etiology. The infantile form so frequent in India probably has its own specific cause.   A number of well known microorganisms have been isolated from the liver in occidental forms of the disease, though the readiness with which infection supervenes on bile stagnation leads one to doubt

[23] Kaufmann, E., Lehrbuch der speziellen pathologischen Anatomie, Berlin, 1907.
[24] Lereboullet, P., Les cirrhoses biliaires, Thèse de Paris, 1902.
[25] Kretz R., *Wien. klin. Woch.*. 1900, xiii, 271.

that their rôle is primary. Hanot's early hypothesis of a primary bile stagnation as the result of a "biliary diathesis" seems to us worthy of reconsideration in certain cases. The essential chronicity of the changes accords well with the relatively innocuous character of human bile; while the enlargement of the spleen which is sometimes great, and the occasional familial character of the malady are facts suggesting an inherent peculiarity of the patient closely akin to that of congenital familial jaundice, as several authors have not failed to point out. Yet it should be remarked in this connection that cirrhosis is not a feature of the latter disease, though for years the liver secretes a tenacious, heavily pigmented bile. Furthermore, we have been unable to cause liver injury in the rabbit by many times increasing the bile output of pigment from small portions of parenchyma (Experiment 3).

The possibility should be borne in mind that local bile stasis may act to complicate any chronic liver derangement in which bile passages, small or large, are compromised. That obstruction to the smaller channels may be responsible for certain of the supposedly "unobstructive" jaundices has been proved by Eppinger. Yet the absence of clinical jaundice is, as we have found, compatible with a condition of total obstruction in more than three-fourths of the liver of the rabbit. Should the same hold true for the human liver the existence of a *"cirrhosis biliaire anicterique"* such as has been described by French authors will not be difficult to understand.

SUMMARY.

There are excellent reasons for employing the rabbit in an experimental analysis of the biliary factor in liver lesions; and it is possible to obtain in this animal results uncomplicated by infection or by intercurrent cirrhosis.

Ligation of the common duct of the rabbit results in a mixed lesion from injury throughout the entire length of the bile channels. By obstructing single ducts and altering the portal stream we have produced cirrhoses of pure monolobular and diffusely intralobular types. The character of the connective tissue changes is determined by the path of escape of bile from the collecting system, which in turn is

largely conditional upon the secretory activity, while this again is dependent upon blood flow. The portal flow is largely diverted from regions of local stasis through encroachment on the stream bed by the dilated ducts.

There is a large margin of safety in bile elimination by the normal hepatic tissue. Less than a quarter of the liver of the rabbit, and this deprived of its entire portal stream, will suffice to keep the organism free from clinical jaundice and healthy when the remainder of the liver, which receives all of the portal blood, has its ducts ligated. The vicarious elimination thus illustrated is of great importance for regions of local stasis by keeping the blood relatively free from bile, thus preventing resecretion into such regions and facilitating exchange from them into the body fluids.

Our experimental monolobular and intralobular cirrhoses are the result of the limitation of biliary lesions to special levels of the duct system. Their resemblance to the different forms of "biliary" cirrhosis associated with Hanot's name is close, and the diverse liver lesions of Hanot's disease are readily explained on the assumption that the stasis, with or without infection, which is indubitably here present, has its situation at different levels in different cases. There are reasons for the view that bile stasis *per se* may sometimes be a prime cause of the malady. Certainly such stasis must be thought of as acting to complicate many chronic liver lesions.

In a later paper experiments on the dog will be described essentially similar in result to those on the rabbit as here set forth.

EXPLANATION OF PLATES.

PLATE 17.

FIGS. 1 and 2. The lesions after 14 days occlusion of the bile duct from the main liver mass (Fig. 1), and after 22 days of such occlusion plus ligation of the corresponding portal trunk (Fig. 2). There is an identical interlobular cirrhosis in both cases with many new-formed bile ducts, simple atrophy of the parenchyma, and a dwindling in size of the lobules. The scattered dark spots are pigmented Kupffer cells such as are found after any local portal obstruction. Hematoxylin and eosin.

PLATE 18.

FIG. 3. Condition of the lobe mass of the liver 2 days after the operation to produce hypertrophic cirrhosis. Necrotic parenchymal cells can be seen here and there. Two are indicated by arrows. The lacunæ near the center of the

lobule on the left indicate where bile thrombi have been dissolved out by the fixative.   Eosin and methylene blue.

FIG. 4. Experimental hypertrophic cirrhosis after 13 days.   In the center of the field is an unusually well preserved lobule with fibroblasts penetrating it from all sides.   Elsewhere only irregular cell cords can be distinguished amid the cirrhotic tissue.   Hematoxylin and Van Gieson's stain.

PLATE 19.

FIG. 5. Experimental hypertrophic cirrhosis after 30 days.   The parenchyma is almost entirely replaced by connective tissue containing many new-formed bile ducts.   The position of the lobules can no longer be made out save from the central veins.   One of the latter is here shown with some degenerating liver cords near it, while elsewhere a few parenchymal cells can be seen.   Eosin and methylene blue.

FIG. 6. Pigment accumulation in the lobe mass 7 days after diversion to it of the entire portal stream and ligation of the common duct.   Masses of bilirubin are to be seen, especially near the center of the lobules.   There is a beginning cirrhosis.   Fresh specimen stained with methylene blue.

PLATE 20.

FIGS. 7 and 8. The lesions 7 days after diversion of the whole portal stream to the lobe mass and ligation of the common duct.   In Fig. 7, of the lobe mass, one finds a punctate parenchymal necrosis which is far more pronounced than when biliary obstruction is local (Fig. 3), while the number of bile thrombi, as indicated by lacunæ, is much greater.   In the main liver (Fig. 8) necrosis takes a gross form although the other signs of stasis are slight.   Both specimens have the same magnification.   The cells of the lobe mass are relatively very large.   Eosin and methylene blue.

PLATE 21.

FIG. 9. Experimental hypertrophic cirrhosis after 21 days.   The fleshy, pink mass with slightly roughened surface next to the pylorus is the cirrhotic lobe mass, well nigh bloodless owing to the obstruction of its vessels.   The spleen, in chronic passive congestion, is a much enlarged, tense cylinder, despite the collateral vein running from it to the main liver.   There was marked ascites in this instance.

PLATE 22.

FIG. 10. Results of 7 days diversion of the portal stream to the lobe mass of the liver with ligation of the common duct.   The lobe mass is enlarged and deeply jaundiced, but without evident necroses, whereas the main liver, somewhat smaller than normal and almost unjaundiced, shows large biliary infarcts.
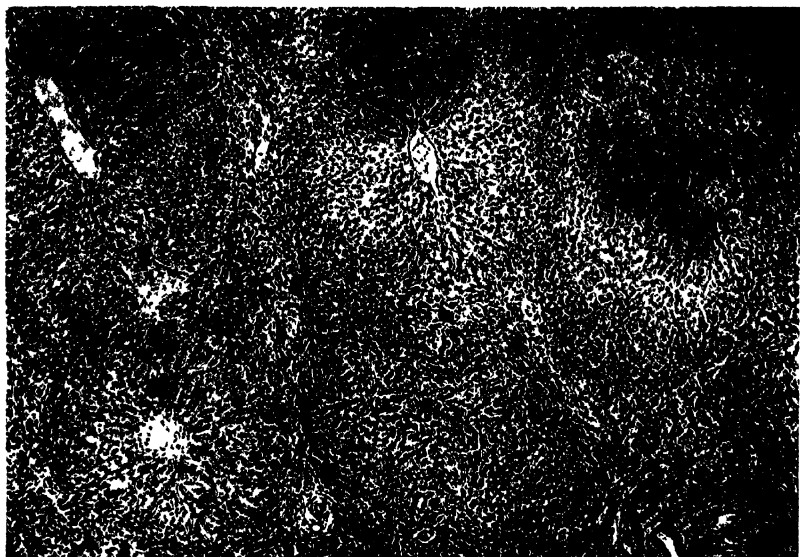
FIG. 1.
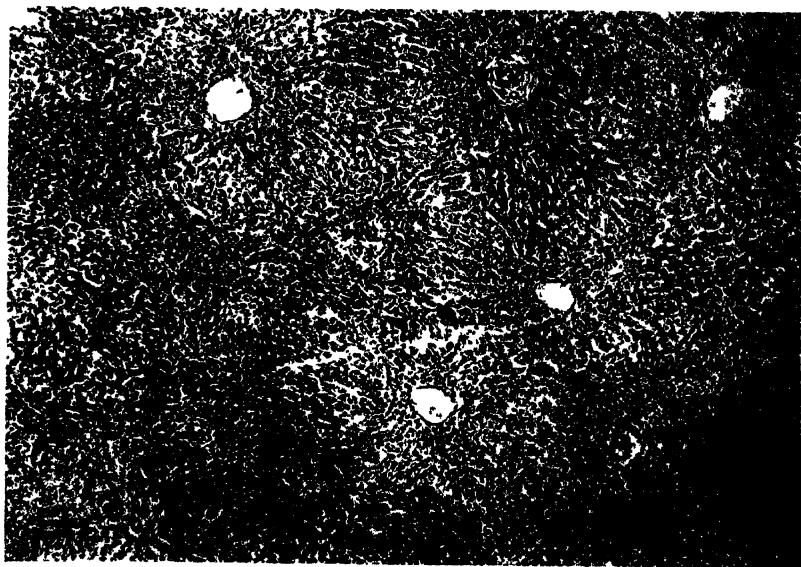
FIG. 2

(Rous and Larimore The biliary factor in liver lesions)
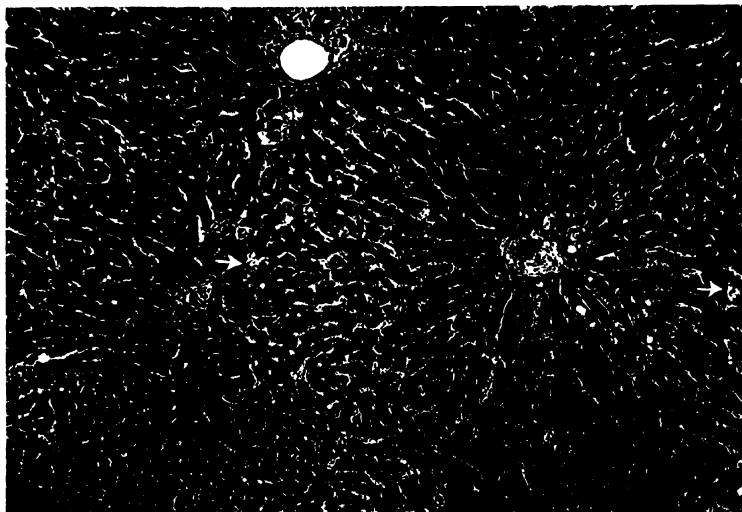
FIG. 3.
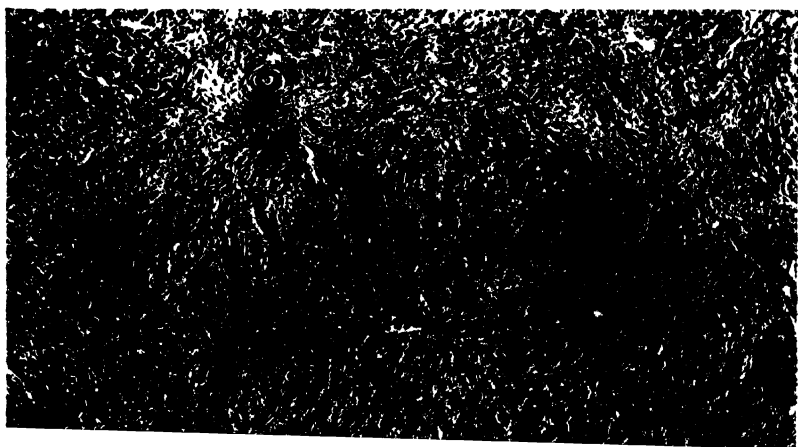
FIG. 4.

(Rous and Larimore: The biliary factor in liver lesions.)

FIG. 5.
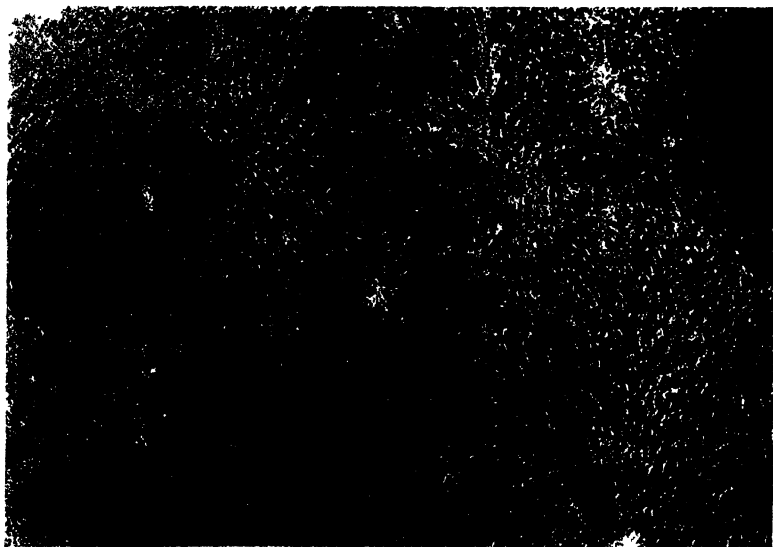
FIG. 6

(Rous and Larimore: The biliary factor in liver lesions.)
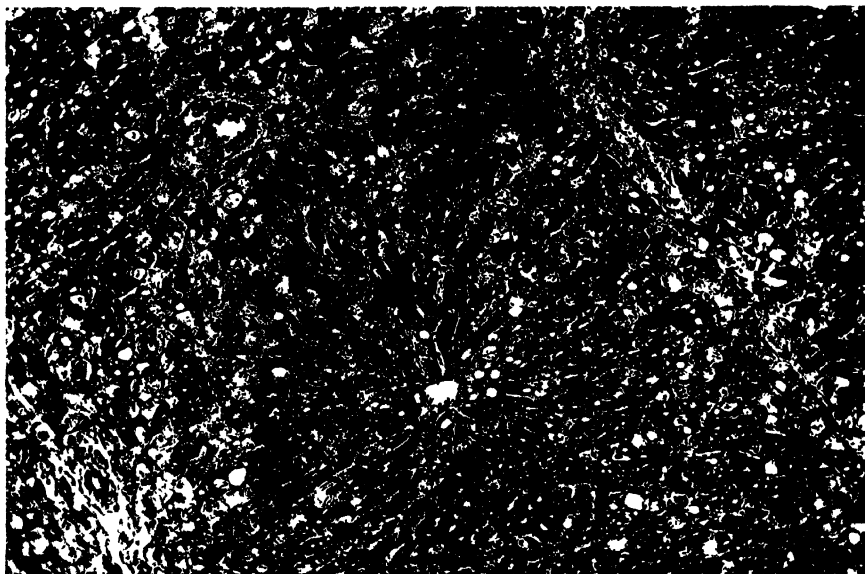
FIG. 7.
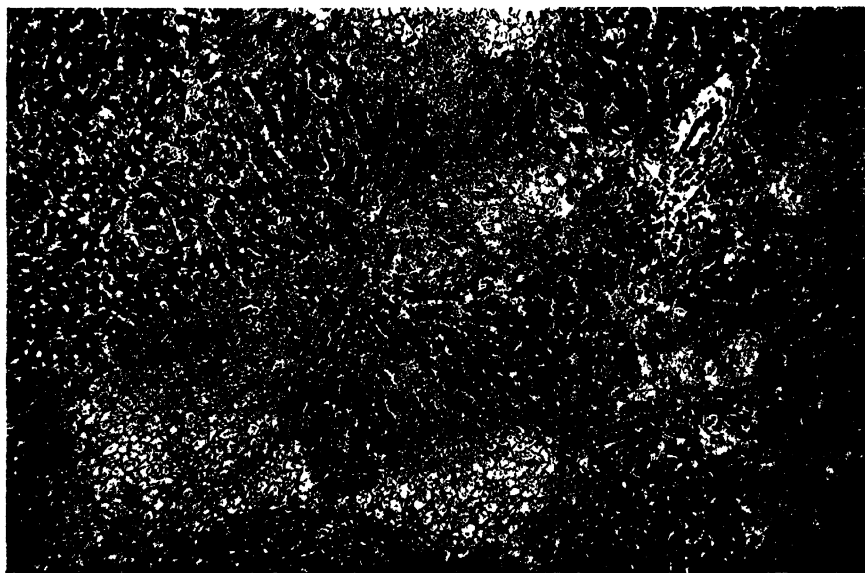
FIG. 8.

(Rous and Larimore: The biliary factor in liver lesions)

PLATE 21

FIG 9

USE OF THE SINGLE CELL METHOD IN OBTAINING PURE CULTURES OF ANAEROBES.

BY M. A. BARBER, PH.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATE 23.

The object of this work has been to determine to what extent the pipette method may be of service in the isolation of anaerobes. No question is raised as to the possibility of isolating anaerobes by plating or by any other of the methods ordinarily in use, but such methods are often time-consuming and sometimes leave the worker in doubt as to whether or not the culture is absolutely pure. Difficulties in isolation are especially great with mixtures of certain anaerobes, and doubt has been thrown on the results of some of the earlier workers because of the probability that their cultures were impure. The pipette method is relatively simple and rapid, and affords the worker the satisfaction of knowing that his culture is the progeny of a single cell. Further, it offers some advantages in the study of anaerobiosis, variability, motility, rate of growth, and behavior in a medium of small sowings exactly known.

Technique.

The technique followed in this work has been essentially that described for aerobes, except that the isolated organisms have been subsequently grown under anaerobic conditions. The pipette method has been fully described (1), and only such details will be given here as should be especially emphasized or are especially applicable to work with anaerobes.

The size of the isolation chamber may vary according to the needs of the investigator, but I have found most convenient one about 7 cm. long, 3.25 cm. broad, and 2 cm. high, which gives abundant working room and allows the use of a large cover-glass, 60 by 35 mm. The

preparation of the cover-glass is a matter of considerable importance, especially when colonies are to be grown on it. Carefully cleaned covers are smeared with vaseline and kept in stock ready for use. Just before use most of the vaseline is removed by holding the cover slantwise under the hot water tap. It is then washed with alcohol and again held under the tap. Finally, it is covered with alcohol and wiped clean with a cloth. It is then perfectly clear to the eye, but retains the very small film of vaseline so necessary to the preparation of small droplets. Prepared cover-glasses may be sterilized by dry heat or over the flame, care being taken to avoid heat sufficient to burn off the vaseline.

The point of the pipette should be made with an opening large enough to admit organisms readily, but not so large as to make it difficult to isolate the organisms in very small droplets. A diameter about equal to the average length of a spore-containing bacillus of *Bacillus tetani* is very satisfactory. It will be found most convenient to make very fine pointed closed pipettes and to break off portions of the point in a drop of broth under microscopic control until an opening of convenient size is obtained. A small quantity of broth should be allowed to enter the pipette before isolation is begun.

Certain doubts which have been raised as to the possibility of isolating a single organism in a hanging drop must arise from a misapprehension of the pipette method (2). The droplets containing the single organism may be made very small, and a mere film of liquid is present on the cover-glass, not a large rounded drop in the margins of which a second organism may lie concealed. The photomicrograph of a spore-bearing bacillus of *Bacillus tertius* (Fig. 1) illustrates the character of the droplet. This droplet has spread somewhat, owing to moisture condensed during the process of photography, and the small particles seen in it are probably due to the excess of vaseline used in the preparation of a specimen suitable for longer preservation during photography. In routine work the organism may be isolated in perfectly clear fluid and in droplets somewhat smaller than that shown in the illustration. After a series of organisms has been isolated, each droplet may be gone over with the oil immersion lens while the cover is on the isolation chamber, or, if the light there is insufficient, when temporarily removed to a shallow moist chamber. One cannot, of

course, exclude the possibility of the presence of any organism not visible to the oil immersion lens, but this limitation is common to all methods of isolating living organisms. If desired, the organism may be washed and rewashed in sterile broth, a procedure useful in removing any growth substances brought over from the original culture, but no guarantee against the inclusion of an invisible living substance. One may observe the first stages of development of an organism in a small drop of broth or at the margin of an agar droplet. In Fig. 2 a young colony of *Bacillus sporogenes* is shown growing out from the original position of a spore at the margin of an agar droplet.

The isolated organisms may be immediately transferred to test-tubes or grown on the cover-glass until colonies have formed and these then transferred. In transferring the organisms immediately after isolation the isolating pipette is discarded, and by means of new pipettes—a fresh one for each organism—the organisms are separately transferred to test-tubes. Each new pipette is supplied with a small amount of sterile broth before use, preferably from recently boiled broth or semifluid agar. In taking up an organism it is important to make sure that it enters the pipette and does not remain clinging to the margin of the opening. This precaution is not absolutely necessary in the case of aerobes or of anaerobes which are transferred to a liquid which is subsequently freed from air, but when a transfer to deep agar or to broth protected by a layer of vaseline or oil is intended, an organism not well within the pipette may remain on the vaseline or at the surface of the agar. In order to provide sufficient liquid for washing the organism well back a very small drop of broth may be made with the pipette in the immediate vicinity of the isolated organism and a small amount of broth added to the drop containing the isolated spore or bacillus just before it is taken up. The organism is then usually seen to enter the mouth of the pipette, and the additional droplet of broth is taken up in order to wash it well back. Sometimes a little manipulation is required to free an organism from substances which tend to make it cling to the film of surface tension of the droplet. Most bacilli and spores still enclosed in the mother cell give little trouble in this respect. When the tip of the pipette is brought to the desired depth in the medium in the test-tube, enough broth should be discharged to insure the

exit of the organism, and the discharge should be stopped before air is introduced.

With bacilli of some species it is desirable to minimize their exposure to the air during isolation. In this case it is well to introduce the isolating pipette directly into the culture from which isolations are to be made. The pipette is adjusted in the holder, a few isolations are made, and the organisms are transferred to an anaerobic environment as quickly as new pipettes can be prepared. Exposure to the air can be limited to 4 or 5 minutes, and the time of exposure can be fairly accurately measured. When pipettes for transferring the isolated organism are made in advance, or when two pipettes are used simultaneously, one for isolating and the other for taking up the organism, the time of exposure to air may be still further limited. No diluting fluid is needed in making isolations if bacilli are taken from a young culture just becoming cloudy.

Seeding Material.

In this work the aim has been to purify cultures isolated by the usual means. It might be possible to use the pipette method in isolating directly from the original source in cases in which the spores of anaerobes predominate and a considerable proportion of them are viable; but, in any case, it would be more convenient to make a preliminary isolation by one of the ordinary means. Spores for seeding were usually taken at maturity or within a few days afterwards, but in some instances spores from cultures 2 to 4 months old were sown and a fair proportion of positive results was obtained. In order to obtain proper bacilli for isolation, media were fairly heavily seeded, and the isolations made at the time when the first cloudiness appeared, usually within 4 or 5 hours. At this period nearly all the bacilli are living, and the longer elements or filaments which are then usually present are more easily isolated and possibly give a better chance of growth. In a few cases positive results were obtained with bacilli from cultures 24 hours old or more. In this paper the term "single bacillus" is made to include, in some cases, short filaments which may have been composed of several united bacilli.

Anaerobiosis.

In part of our series (Tables I and II, columns marked*) organisms were transferred to a fluid medium and the air was subsequently removed by means of an air pump. In the majority of cases boiling alone without vacuum was relied on to remove oxygen, and with

TABLE I.

Growth of Single Bacilli in Test-Tubes.

Species.	Glucose broth.	Serum glucose broth.	Semisolid glucose agar.	Semisolid serum glucose agar.	Semisolid serum agar.	Firm glucose agar.	Firm Veillon agar.	Liver peptone agar.	Minced brain.	Liver peptone water under vaseline.	Liver peptone water in vacuum.*	Milk.*	Plain broth.*	Total, all media.
B. *sporogenes*	2/14					0/6								2/20
" *welchii*	10/25		0/3						0/1	0/7	3/6	1/3	0/1	14/46
" *tetani*	13/36		3/12				1/3				0/5			17/56
" *œdematis*	3/12													3/12
" *botulinus*	3/6						1/3				0/5			4/14
B. of Ghon Sachs.	0/16		1/7			0/1		0/6		1/6				2/36
B. *aerofœtidus*	1/3	0/1	1/2								0/23			2/29
" *putrificus*	0/2	1/2		1/3										2/7
" *bellonensis*	0/3	0/3	1/2	0/3										1/11
" *tertius*	0/1	2/3	0/2	0/2	3/3									5/11
" *fallax*	0/8			0/2	1/2									1/12
" *œdematiens*	2/3	0/2	1/1											3/6
" *bifermentans*	1/12	0/1	0/1	0/1	0/4					0/1				1/20
" *histolyticus*	1/1			1/3						2/2				4/6
Vibrion septique	0/47	0/8	1/37	0/12	0/7					0/3				1/114
Total, all species.	36/189	3/20	8/67	2/26	4/16	0/7	2/6	0/6	0/1	3/19	3/39	1/3	0/1	62/400

* Air removed after inoculation by means of an air pump.

fluid media access of air was prevented by a layer of vaseline 1.5 or 2 cm. thick. Immediately before inoculation the upper part of the vaseline layer was liquefied by gentle heat. Both firm and semisolid agar was used without any layer of vaseline or other protecting substance, and the isolated organism was introduced deep into the medium (Fig. 3). Except with serum-containing media, tubes were, as a routine, boiled and quickly cooled before use.

A simple method of anaerobiosis was found adequate for growing spores in hanging drops. A shallow moist chamber about 45 mm. long, 25 mm. broad, and 2 mm. deep, inside measurement, is made by cementing strips of glass to a large slide. Organisms are isolated in an area of about 3 by 1 cm. in the center of a large cover-glass. The isolated spores are arranged about 1.5 or 2 mm. apart, and enough medium is added after isolation to provide each spore with a droplet

TABLE II.

Growth of Single Spores in Test-Tubes.

Species.	Medium and proportion of positives.													Total, all media.
	Glucose broth.	Serum glucose broth.	Semisolid glucose agar.	Semisolid serum glucose agar.	Semisolid serum agar.	Firm glucose agar.	Firm Veillon agar.	Liver peptone water under vaseline.*	Liver peptone water in vacuum.*	Milk.*	Plain broth.*	Egg cube.*	Meat.*	
B. sporogenes.......	11/16	2/5		3/6		2/5		5/6		1/1	1/2	1/1		26/42
" tetani...........	7/17		3/6	2/6			0/5		0/14					12/48
" botulinus........							0/3		1/12					1/15
B. of Ghon Sachs...	3/3		1/3											4/6
B. putrificus.......				4/5						1/1	1/1	1/1	1/1	8/9
" bellonensis......		0/2	0/5	4/5	0/3				2/6					6/21
" tertius..........				10/12	1/1				1/7					12/20
" œdematiens......				4/5					3/7					7/12
" bifermentans.....			3/3	4/6					1/3					8/12
" histolyticus......									2/11		0/2			2/13
Vibrion septique....			1/1	3/3			0/4		3/5					7/13
Total, all species.	21/36	2/7	8/18	34/48	1/4	2/5	0/12	5/6	13/65	2/2	2/5	2/2	1/1	93/211

* Air removed after inoculation by means of an air pump.

about 1 mm. in diameter, although this quantity may be made to vary, as desired. Larger drops sown with many organisms are also made for comparison. After the organisms have been isolated and supplied with medium, the under surface of the cover-glass, with the exception of the area in the center occupied by the isolations, is covered with soft glucose agar taken from the surface of a culture of *Bacillus pyocyaneus* about 4 or 5 hours old. This culture should contain many actively growing organisms, and a little fresh medium

may be added to it just before spreading. The area in the center of the cover may be enclosed in a ring of soft paraffin in order to prevent any danger of the spread of the *pyocyaneus* to the isolated anaerobes. The bottom of the moist chamber is supplied with a similar layer of *pyocyaneus* culture. The entire surface of the bottom may be covered, but better light is afforded for observation and there is less danger of condensation of an excess of moisture on the cover-glass if an area at the center of the bottom is left free. The cover is immediately sealed on the moist chamber by means of vaseline, and the chamber is enclosed in a Petri dish before it is placed in the incubator.

Pyrogallic acid and potassium hydroxide, so arranged that they may be mixed in the bottom of the moist chamber after the cover has been sealed on, were used alone and in connection with the *pyocyaneus* culture, but appeared to offer no advantage.

Preparations growing on the cover may be examined at any stage of growth and returned to the incubator without disturbing the cover-glass. Moist chambers taken out of the incubator should not be exposed to room temperature while still warm, since the cover-glass cools more rapidly than the slide and may accumulate an oversupply of moisture of condensation. It is best, then, to allow the preparations to attain room temperature before removing them from the Petri dish.

Media.

The media employed are shown in Tables I and II. Glucose media contained for the most part 0.5 per cent of glucose and were adjusted to a pH of approximately 7.4. Serum media contained 3 to 5 per cent of unheated rabbit serum, or, in a few instances, horse serum. The liver peptone water consisted of Dunham's peptone without salt adjusted to a pH of 7.8. The media were tubed, and to each tube a small piece of liver was added. It was then autoclaved, and after autoclaving the reaction was found to be about pH 7.4. Semi-solid agar media were made soft enough to be easily drawn into a fine pointed pipette while cold. As a routine, test-tubes of narrow diameter were used, and 10 cc. of media supplied to each tube.

Proportion of Positive Results Obtained from One-Cell Sowings in Test-Tubes.

Table I shows the number sown and proportion of positives of single bacilli sown into various media in test-tubes, and Table II gives similar data for spores. The denominators of the fractions in these and all subsequent tables give the number sown; the numerators, the number positive. Fifteen species, or possible species, are included. Questions as to the possible identity of certain species, as the bacillus of Ghon Sachs and *vibrion septique*, need not be discussed here. There were four strains of *Bacillus tetani*, one of them a Type III, two strains of *Bacillus botulinus*, and two of *Bacillus welchii*, one of which had been recently isolated from feces. For the most part the various strains had long been grown on nutrient media. In all strains of all species positive results were obtained either from single spores or from single bacilli, and in a majority of species from both. In the aggregate, 211 sowings of spores gave 44.1 per cent positive, while 400 sowings of bacilli gave only 15.5 per cent positive. The poorer showing of the sowings of bacilli is mainly due to the large number of negatives occurring in three species, *Bacillus aerofœtidus*, the bacillus of Ghon Sachs, and *vibrion septique*. The last two named gave positives with single spore sowings on the first trial.

As a routine, isolations were done in batches of five or six transfers. In a majority of species the first trial gave at least one positive among the first four tubes inoculated. In nearly 70 per cent of the thirty-six spore groups positives were obtained in at least one of the first four tubes inoculated. Fourteen spore groups, including nine species and ten strains, were sown into semisolid glucose agar, part with and part without serum. Of these, thirteen gave positives in at least one of the first four tubes inoculated. To isolate six spores or bacilli and transfer them to test-tubes requires, as a rule, less than 1 hour. These data give a better idea of the practicability of the method and the probabilities of success than do the percentages given in the tables, which include all experiments, tentative and otherwise.

Hardly enough experiments were made with all the different media to offer a fair basis for estimating their comparative value. The various glucose media served about equally well for bacilli, and semi-

solid serum glucose agar gave the best results for spores. Semi-solid agar media not only give a relatively high proportion of positives, but offer the most convenient form for one-cell inoculations, since they do not need to be liquefied for inoculation and require no protective layer of vaseline or oil. Further, if positives are examined early one may often observe the formation of a single colony at the point where the organism was discharged. Certain advantages of semisolid media for routine work with anaerobes have been described by Lignières (3).

Bacteria in positive cultures in all media were examined microscopically, gas formation and other characteristics noted, and transfers made to broth or other media under aerobic conditions, in order to detect possible contaminations with air organisms. Such contaminations during the manipulations occur so rarely and are so easily detected that they constitute no source of difficulty in the pipette method.

Growth of Isolated Organisms · in Hanging Drops.

The method of anaerobiosis for preparations in hanging drops has been described. Single spores from eight species were successfully grown. The media used were chiefly semisolid glucose agar, with and without serum, and serum glucose broth. The species tested and the proportion positive were as follows: *Bacillus tetani*, 17/57; bacillus of Ghon Sachs, 1/7; *Bacillus sporogenes*, 42/50; *Bacillus putrificus*, 1/8; *Bacillus bellonensis*, 11/24; *Bacillus œdematiens*, 4/12; *Bacillus tertius*, 10/19; *Bacillus bifermentans*, 1/29. Total, 87/206, or 42.2 per cent.

In some batches of all species single spores from the same source were sown at the same time into about 10 cc. of a similar medium in test-tubes. The test-tubes gave a total of 55.4 per cent positives out of 65 sown, while the corresponding hanging drops gave 38.2 per cent positives out of 102 sown.

The hanging drop method has certain advantages; isolations are quickly made—¾ hour or less suffices for a series of ten isolations— and only two pipettes are required for a whole series, one for isolation and one for supplying additional media to the droplets. Early growth, variability, motility, and spore formation may be conveniently observed.

With species which readily form spores, it would probably save time in isolation to sow spores in hanging drops in preference to the direct transfer to test-tubes. The strains which fail to grow on the cover-glass could be reserved for the other method. Lag occurs in the hanging drop as in test-tubes, and preparations should not be discarded if no growth occurs on the 1st day. When colonies have formed in the hanging drop they may be easily transferred to the test-tube. In most species spores formed readily in the hanging drop, and these spores have afforded convenient material for further isolations.

The hanging drop method has not proved successful for growing isolated bacilli, although a large number of bacilli sown in one drop will often grow. A number of attempts to grow young isolated bacilli of several different species gave uniformly negative results, possibly because of too long exposure to air before sufficient anaerobiosis was attained.

Growth of Spores of Bacillus tetani in Homologous Serum.

The growth in hanging drops of single tetanus spores in a medium containing tetanus antitoxin was compared with similar sowings in a control medium containing no antitoxin. Both media consisted of semisolid glucose agar containing 3 per cent horse serum. The antitoxin serum contained approximately 325 units per cc., and the tetanus spores were taken from the strain used in immunization. Growth occurred in three out of ten sowings in the antitoxin medium, and in two out of ten controls. In a series sown in serum glucose broth the antitoxin medium gave one out of four positive, and the control one out of five. Spore formation and the quantity and character of the growth were similar in the two media, except that in the antitoxin medium bacilli showed a greater tendency to adhere in chains. Sowings of single spores in quantities of 10 cc. of the antitoxin medium gave two positive out of three sown. One of these positives is shown in Fig. 3.

Effect of Exposure to Air on Isolated Bacilli and Spores.

In the course of routine work the period of exposure to air was noted in many groups of experiments, and the proportions of positives occurring after various periods of exposure are shown in Table III. Only batches in which at least one positive occurred and only those in which young bacilli were sown are included in this table. The bacteria were exposed to the air in the very small droplets of the

TABLE III.

Effect of Exposure to Air on Young Bacilli.

Species.	Length of time exposed to air and proportion of positives.									Total, all periods.
	4–6 min.	7–10 min	11–17 min.	18–23 min.	24–30 min.	31–36 min.	37–43 min	44–60 min.	61–72 min.	
B. welchii............	3/3	1/2	3/5	1/3	0/4	0/2	0/2			8/21
" tetani..............	3/7	3/4	3/7	3/10	1/4	1/5	1/3	1/7	0/1	16/48
" œdematis...........	2/2	1/1		0/3	0/1	0/2	0/2	0/1		3/12
" botulinus...........	1/1	0/1	1/1	0/2	1/1					3/6
" sporogenes..........	0/1	1/1	0/1	0/1	1/1	0/1				2/6
B. of Ghon Sachs.......	2/2	0/1	0/1	0/1	0/2					2/7
B. aerofœtidus..........	1/3	1/?	0/1							2/6
" putrificus...........	2/3	0/3	0/1							2/7
" bellonensis..........	1/3	0/2								1/5
" tertius..............	2/2	3/3	0/1							5/6
" fallax..............	1/2	0/2	0/2							1/6
" œdematiens..........	1/2	1/2	1/2							3/6
" bifermentans.........	1/3	0/3	0/2							1/8
" histolyticus..........	2/2	2/2	0/2							4/6
Total, all species.....	22/36	13/29	8/26	4/20	3/13	1/10	1/7	1/8	0/1	53/150
Percentage positive...	61.1	44.8	30.8	20.0	23.1	10.0	14.3	12.5	0	35.3

liquid, usually glucose broth, in which they had been grown, and the period of exposure is reckoned from the time when a bacillus issued from the isolating pipette until it was deposited in the test-tube under anaerobic conditions. To the time of full exposure on the cover-glass one should add, theoretically, the time necessary for the reduction of any free oxygen introduced with the organism into the test-tube. However, the time necessary to attain sufficient anaerobiosis for growth must be short, since a very tiny amount of liquid is brought into 10 cc. of medium.

It appears from Table III that, on the whole, the probability of growth of young bacilli decreases with the increase in the time of exposure to the air, and that different species vary somewhat in sensitiveness to oxygen, as far as can be judged from the comparatively small numbers under each.

The results obtained with *vibrion septique* are not included in this table, since a different method of isolation was used for the batch in which a positive was obtained. Nearly 100 trials with sowings of young bacilli of *vibrion septique* were made by the usual method without success. The organisms were living when isolated, since in many cases the bacillus was distinctly motile, although motility ceased after an exposure to the air of $\frac{1}{2}$ or $\frac{3}{4}$ minute. A great variety of media was employed. The addition to glucose broth of salicin, of unheated pieces of rabbit kidney, and of broth from filtered young cultures of *vibrion septique* and of *Bacillus welchii* gave no success. It hardly seems likely that the medium was at fault, since single spores of this species grew readily in semisolid serum glucose agar—in one series of four all were positive. Furthermore, larger sowings of young bacilli grew rapidly in every medium tested. The time of exposure to air was cut down to $1\frac{1}{4}$ to 2 minutes by the employment of transfer pipettes prepared in advance and by the simultaneous use of two pipettes, but no growth was obtained. Sowings of ten or fifteen bacilli after an exposure to the air of 4 to 6 minutes also gave negative results.

Finally, a series of isolations was made under sterile paraffin oil spread on the cover-glass. The first oil series gave one positive out of seven sown. The organism which grew was motile when transferred. A second paraffin oil series of eight transfers gave all negatives, and all of a series of fourteen isolated under vaseline or vaseline diluted with paraffin oil were also negative. Motility persisted somewhat longer under vaseline than in similar droplets exposed to the air. In small hanging drops made in an atmosphere from which oxygen had been removed by pyrogallic acid and potassium hydroxide motility persisted for an hour or more.

It seems probable that young bacilli of *vibrion septique* are especially sensitive to free oxygen, and that this sensitiveness explains in large part the negative results obtained after the transfer of single bacilli.

Our experiments tend to confirm those of Bachmann (4), who found the vegetative forms of certain anaerobes very sensitive to an exposure to air of only 10 minutes plus the time required to obtain full anaerobiosis subsequently. In Bachmann's experiments bacilli were exposed to the air in agar plates, and the plates were subsequently brought under anaerobic conditions in which organisms remaining viable might form colonies.

In our experiments spores showed no reduction in the proportion of positives as the result of exposure to air during periods as great as 85 minutes at least. In 61 one-spore sowings, in which the time of exposure to air was noted, the periods of exposure and the proportion of positives were as follows: 12 to 17 minutes, 2/3; 18 to 23 minutes, 2/6; 24 to 30 minutes, 6/9; 31 to 36 minutes, 6/8; 37 to 43 minutes, 5/9; 44 to 60 minutes, 6/10; 61 to 72 minutes, 4/10; 73 to 85 minutes, 3/6. Total for all periods, 34/61. These results represent the aggregate of six species and include only batches in which at least one positive occurred.

The effect of exposure to air on anaerobes was not made the subject of a special study in our experiments, but only as incidental to the technique of isolation. It would appear that in the isolation of bacilli one should minimize the exposure to air as far as possible, while the viability of spores is not appreciably affected by an exposure of an hour or more.

Lag.

In a great majority of the experiments the inoculated test-tubes were not enclosed in an anaerobic jar, and the time of first appearance of growth could be approximately noted. In all positives occurring in tubes in which bacilli were sown, growth appeared on the day following inoculation. The first appearance of cloudiness in broth or of a colony in agar varied approximately from 16 to 21 hours after sowing. Growth from single spore sowings showed a marked tendency to lag. The amount of lag by days is shown in Table IV. A day was reckoned to the time of the last observation, usually about 5 p.m. If growth appeared on the 1st day, it was usually later than in sowings of single bacilli of the same species. It is seen from Table IV that about 37 per cent of the positives appeared on the 2nd day or later,

thus showing a lag of a day or more.   The condition of the spores sown is doubtless an important factor.   This fact is illustrated by the results with the washed spores of *Bacillus tertius*.   Spores were twice washed by centrifugation in normal salt solution, and two of the three positives lagged 3 days or more.   Three grew out of a series of five sown.   Unwashed spores from the same source gave all positives, and all grew without lag.   In addition to the washing the spores were kept several hours in salt solution at refrigerator temperature.

<div align="center">TABLE IV.</div>

Time of Appearance of Growth from Single Spores Sown in Various Media in Test-Tubes.

Species.	Total No. of positives.	Day following inoculation on which growth appeared and incidence of positives.						
		1	2	3	4	5	6	8
B. sporogenes...................	20	6	12	2				
" tetani......................	12	9	2		1			
" putrificus..................	6	4	2					
B. of Ghon Sachs..............	4	4						
B. bellonensis.................	4				2		1	1
" œdematiens.................	4	4						
" tertius (unwashed spores).....	8	8						
" " (washed ").....	3	1			1	1		
" bifermentans................	7	7						
Vibrion septique...............	4	2		1	1			
Total......................	72	45	16	3	5	1	1	1

The marked tendency to latency observed in the growth of the spores of some species illustrates one source of difficulty in separating these from other species by plate methods.

Variability in Morphology of Bacillus sporogenes.

The method employed in isolation is available for a study of variability in anaerobes as in aerobes, as is shown by the following experiment.   Four spores from a culture of one-cell origin were isolated and sown in hanging drops.   Of the four colonies formed, one showed a marked tendency to the formation of filaments, these appearing in the form of a fine network.   Six spores from the filamentous colony

were isolated in a new series of hanging drops, and all showed the same filamentous tendency to a greater or less degree as compared with a series of controls from a non-filamentous source. In further series on a different medium the filamentous strain reverted to normal. A long filament isolated and transferred directly to a test-tube gave the same type of growth as controls.

DISCUSSION.

It is a matter of common knowledge that large sowings of bacteria into a new medium give a better chance of growth than small sowings. Large sowings offer a larger assortment of individuals, some of which may be more vigorous or otherwise better capable of adapting themselves to new conditions, and, furthermore, any growth products carried over with the bacteria may favorably modify the new environment. When a single cell is sown the change in environment is more complete, and unless the new environment is identical with the old a process of adaptation may be necessary. In the experience of the writer, single aerobes have often given 100 per cent of positives when sown into as much as 10 cc. or more of new medium favorable to the species sown.

The importance of employing a suitable medium is shown by experiments on Pneumococcus Type I. Two batches of plain broth made during successive weeks and apparently similar in constituents and reaction were compared. Six tubes of Batch 1 and six of Batch 2 were alternately sown with pneumococcus taken at the height of growth. Tubes of Batch 1 received each one pair of pneumococcus, and those of Batch 2 received each one to four pairs. All the tubes of Batch 1 showed abundant growth which proved to be pure culture of pneumococcus, while all the tubes of Batch 2 remained sterile. All the tubes contained 10 cc. of medium. A flask containing 75 cc. of Broth 1 also gave an abundant growth with a sowing of one pair. Large sowings in Broth 2 grew readily.

When single anaerobes are sown, the lack of proper conditions of anaerobiosis may offer more obstacles to growth than is the case when larger sowings are employed. Burri and Kürsteiner (5) have shown that the growth of *Bacillus putrificus* initiated under strict anaerobic

conditions may continue with increased activity under conditions of aerobiosis unfavorable to the beginning of growth.

In our experiments 100 per cent of positives were obtained with certain lots of five or six tubes each in which single spores of *Bacillus tertius*, *Bacillus sporogenes*, and *Bacillus bifermentans* were sown. Single spores from a 48 hour culture of *Bacillus subtilis* gave all positives in six tubes of glucose broth with a vaseline layer. The medium was the same as that used for anaerobes except that the free oxygen was not expelled by heat. These results would indicate that the technique of the transfer of the single organism is not at fault, and that failures to grow should be ascribed to a lack of viability of the organism or to its failure to adapt itself to a new environment. The viability of anaerobic bacilli is affected by exposure to air, a fact which explains in part the small proportion of positives as compared with single bacilli or cocci of aerobes. The negatives in spore sowings can hardly be due to lack of proper conditions of anaerobiosis, since spores are little sensitive to short exposures to air, and abundant time is available for the reduction in the new medium of the small quantity of free oxygen introduced with the spore. The quantity of medium to which the spore is transferred can hardly be an important factor in itself, since spores sown in small hanging drops give a smaller proportion of positives than when sown into test-tubes. The quality of the medium is probably more often the determining factor, and if viable spores and a medium exactly suited to each species are employed, all, or practically all the spores might be expected to grow. The process by which an organism adapts itself to a new and less favorable medium is imperfectly known. Our experiments indicate that organisms apparently similar vary greatly in their power of adaptation.

SUMMARY.

The pipette method has proved a feasible method of obtaining one-cell pure cultures of anaerobes.

Both bacilli and spores may be used as seeding material, but spores give a much higher percentage of positives.

Boiling alone affords a sufficient degree of anaerobiosis to the medium for initiating one-cell growths, and semisolid agar is the most convenient form of medium.

Exposure to air during isolation apparently has no effect on the viability of spores of anaerobes, but young bacilli of some species suffer from a comparatively short exposure to free oxygen.

I wish to thank Colonel F. F. Russell, in charge of the Army Medical School at Washington, where a portion of this work was done, and Major Benjamin Jablons, recently in charge of the work on anaerobic bacteria in the Army Medical School, for many courtesies shown and for a large proportion of the cultures of anaerobes used in these experiments. I am also indebted to Dr. Charles Krumwiede, of the Research Laboratories of the Department of Health of the City of New York, for a quantity of tetanus antitoxin.

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5. Burri, R., and Kürsteiner, J., *Centr. Bakteriol.*, 2te Abt., 1908, xxi, 289.

EXPLANATION OF PLATE 23.

FIG. 1. Spore-bearing bacillus of *B. tertius* isolated in a hanging drop of broth. × 1,000.

FIG. 2. Colony of *B. sporogenes* grown from a single spore in a hanging drop of semisolid glucose agar. × 120.

FIG. 3. Colony of *B. tetani* grown from a single spore in glucose agar containing tetanus antitoxin. About natural size.
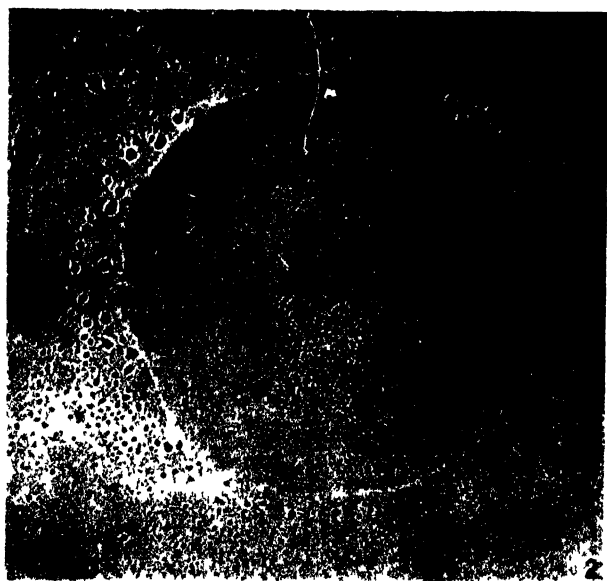
ARE THE SUPERIOR CERVICAL GANGLIA INDISPENSABLE TO THE MAINTENANCE OF LIFE?

BY S. J. MELTZER.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

Read before the Academy, April 26, 1920.

The sympathetic nerve fibres which originate in the thoracic part of the spinal cord terminate in the superior cervical ganglia, from which separate bundles of nerve fibres go to the various parts of the head. Experiments with stimulation of the central end of the cervical sympathetic nerves revealed that these nerve fibres control various functions localized in the head. For instance, to mention the more important ones, the contraction and dilatation of the bloodvessels of various parts of the head, the constriction and dilatation of the iris and the secretion of various glands. Section of the sympathetic nerve showed that some of these innervations are in a state of tonus, for instance, the pupils become constricted and the bloodvessels of the ear (to be seen best in rabbits) become dilated. By means of local application of nicotine to the ganglia which paralyzes postganglionic but does not affect preganglionic nerve fibres, Langley has shown that some of the postganglionic nerve fibres of the superior cervical ganglia are simply a continuation of the cervical sympathetic, preganglionic nerve fibres, while other postganglionic nerve fibres have their origin in nerve cells within the superior cervical ganglia.

About sixteen years ago we[1] showed that epinephrin (adrenalin) which, if administered by subcutaneous injection, or by instillation

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[1] Meltzer, S. J., and Auer, Clara Meltzer, "Studies on the Paradoxical Pupil-Dilatation Caused by Adrenalin. I.—The Effect of Subcutaneous Injections and Instillations of Adrenalin upon the Pupils of Rabbits," *Amer. J. Physiol.*, 11, 1904 (28–36); Meltzer, S. J., "Studies on the Paradoxical Pupil-Dilatation Caused by Adrenalin. II.—On the Influence of Subcutaneous Injections of Adrenalin upon the Eyes of Cats after Removal of Superior Cervical Ganglion," *Ibid.*, 11, 1904 (37–39); Meltzer, S. J., and Auer, Clara Meltzer, "Studies on the Paradoxical Pupil-Dilatation Caused by Adrenalin. III.—A Discussion of the Nature of the Paradoxical Pupil-Dilatation Caused by Adrenalin."

in the conjunctival sac, in normal animals, shows no influence upon the size of the pupil, produces promptly a dilatation of the pupil and a considerable reduction of its responsiveness to light, if the subcutaneous injection or the instillation were made in animals in which the corresponding ganglion was removed. To obtain this result the subcutaneous injection or instillation must be made about twenty-four hours in rabbits and forty-eight hours in cats and dogs after the removal of the ganglion. Cutting of the cervical sympathetic nerves has no such effect. These facts were best illustrated in an experiment in which the superior cervical ganglion was removed on one side and the sympathetic nerve was cut on the other side. A simultaneous instillation of adrenalin in both conjunctival sacs caused a dilatation of the pupil on the side in which the ganglion was removed, but not on the side in which merely the sympathetic nerve was cut. For that reason we designated this pupilary phenomenon as "paradoxical." These experiments revealed that in the normal state, and for twenty-four hours in rabbits, or for forty-eight hours in cats and dogs, after the removal of the superior cervical ganglion, the normal condition of the pupil is maintained by influences which take their origin in nerve cells within the superior cervical ganglion and not in the spinal cord.

In studying at various times certain phases of this experiment, I was sometimes annoyed by failures which were caused by early deaths of the animals. A survey of these failures revealed the fact that they took place in animals in which both ganglia were removed. A survey of the several changes in the functions which stimulation of the sympathetic nerves or section of these nerves bring about, shows that these changes, interesting as they are, affect only peripheral conditions, and do not endanger life. But some of the postganglionic fibres of the superior cervical ganglion enter the "brain" before they reach the periphery. May not some of these fibres also terminate in some vital point within the brain or the medulla? It is true, in our previous experimental studies, death of the animal was rather an exception. But these studies were essentially confined to the removal of a ganglion on one side only. From experiments upon the vagus nerves we know that cutting of one vagus does not affect the life of the otherwise normal animal, while, when both vagi are cut, with few exceptions, the animal dies within a day or two after the operation.

May we not meet with a similar result by the removal of both ganglia? I therefore started a series of experiments in which both superior cervical ganglia were removed.

This series of experiments was made chiefly on rabbits. Although in these animals the superior cervical ganglion is small, it offers the advantage that it is not intimately connected with the vagus nerve, as is the case in dogs and cats; the ganglion in rabbits may therefore be readily removed without causing injury to the neighboring vagus nerve. (It is not superfluous to state that the present series of experiments was carried out last winter, beginning November 3rd.) To this date more than eighty rabbits were used for the study of various phases of our problem. I shall state the results briefly.

For the chief issue of our problem we used twenty-eight animals in which both superior cervical ganglia were simultaneously removed under ether anesthesia. Of these, twenty-five died as follows: Six died in less than twenty-four hours, that is, the animal which was operated before noon or in the afternoon was found dead next morning at about nine o'clock. Nine animals died in less than two days, five in less than three days, one in four, one in five, one in six, one in eight and one in ten days. The autopsies revealed the presence of pulmonary lesions in all these animals. Three animals survived the removal of the ganglia and were killed after many weeks or months and no pulmonary lesions were found.

Both ganglia were also removed in two cats which died in less than two days and showed pulmonary lesions.

At an early stage of the investigation we met with one failure in a cat; it survived for many days the removal of both ganglia. Suspecting that the operation might not have been successful, it occurred to me, that this might be tested by the action of adrenalin upon the pupil. In the experiments with adrenalin,[2] it was observed that when a ganglion was incompletely removed, instillation or subcutaneous injection of adrenalin did not cause the characteristic upon the pupil. I therefore applied this method to the surviving cat and found indeed that an instillation in a conjunctival sac as well as a subcutaneous injection of adrenalin neither caused a dilatation of the pupil

[2] Meltzer, S. J., and Auer, Clara Meltzer, L. c. (30).

nor a retraction of the nictitating membrane, the latter being one of the striking effects of adrenalin in ganglionectomized cats. The animal was then killed on the tenth day after the operation, and it was established that neither of the ganglia were successfully removed, and that the lungs showed no lesions. Also in one surviving rabbit it was found that adrenalin caused no dilatation of the pupil of the right eye. The autopsy showed normal lungs, proved that the ganglion on the right side was only partly removed. Henceforth an adrenalin test was made on the pupils of practically all animals in which the ganglia were removed. This was also done in the above mentioned three rabbits in which the removal of the ganglia did not lead to the death of the animals. In one of these animals the effect of adrenalin was indeed insignificant; in the other two rabbits, however, the instillation of adrenalin brought a definite dilatation of both pupils. In some rabbits the removal was purposely restricted to one half, or a little more than one half, of one ganglion or of both. As a rule the part to be removed was first crushed and then torn away. In these cases the adrenalin test was made a day or two later; the result was negative, and the animal survived even when both ganglia were mutilated.

However, in testing the success of the operation by the use of adrenalin, it has to be borne in mind that while a negative result proves that the corresponding ganglion was incompletely removed, a positive adrenalin test does not necessarily prove that the ganglia were so completely removed as to bring about the death of the animal. It may be that the number of nerve cells the removal of which is sufficient to bring about a positive adrenalin reaction upon the pupil, is smaller than the number of cells which have to be removed in order to bring about the death of an animal. In other words, the presence and integrity of a small number of nerve cells may be sufficient to maintain life, while the balance of the cells serve only as *factors of safety*—a condition which is especially met with in glands with internal secretion. It follows that in the above mentioned surviving rabbits in which the adrenalin test was positive, we had no assurance that the ganglia were so completely removed as to bring about the death of these animals. (It has to be added that at the autopsy of the three animals neither a minute macroscopical nor a microscopical search was made for possible remnants of the ganglia.)

However this may be, *this series of experiments showed that in about ninety per cent of the animals, the operative removal of both ganglia proved to be fatal to the animals—an experiment which, as far as I know, was never made before.* The question now arose: Definite as the result is, is it important, that is, does it prove that the ganglia are really important to the maintenance of life, or was the fatal outcome perhaps due only to the operative procedure and not to the indispensability of some principle provided by the ganglia? Pulmonary lesions were found at the autopsy after the removal of both ganglia; pulmonary lesions are found after cutting both vagi. The ganglia are in the very proximity of the vagus nerves. May not the operative procedure injure both vagi sufficiently to bring about a "vagus pneumonia?"

The following considerations and experimental facts speak against such an interpretation. The possibility of injuring the vagus nerves was taken into consideration at the very beginning of this study. As it was stated before, it was just on account of this possibility that rabbits were given the preference. In these animals the superior cervical ganglion is separate from the vagus nerves, and it may be removed without touching the nerves or pulling at them. With this requirement in mind, I tried to carry out the experiment with care. It is not quite probable that I should have failed on both sides in nearly all my attempts, and that the injury which I might inadvertently have afflicted to both vagus nerves should have been of such an extent as to be equal in effect to a complete section of the nerves. Furthermore, in several experiments one of the ganglia could not be found and only one ganglion was removed. In these cases in which a long search for the ganglion was made the tissues and the corresponding vagus nerve had to be quite severely manipulated. The animals nevertheless survived this procedure. Finally in some experiments, the lower half of both ganglia were removed, as mentioned before. Here the lower half was first crushed and then removed, which required more manipulations than the tearing out of the entire ganglion. These animals survived the operation without manifesting any untoward symptoms.

More direct and decisive evidence was obtained by stimulations of the central end of the vagus nerves after removal of the corresponding ganglion, and after removal of both ganglia. The vagus nerves carry

centripetal as well as centrifugal nerve fibres. The centripetal fibres affect and control the respiration and cause a change in the state of blood pressure. Further, the vagus trunk and the superior laryngeal nerve, a branch of the vagus, carry fibres the stimulation of which causes reflex deglutition. The vagus nerve seems to contain also sensory fibres. The stimulation was effected by Faradic currents of various strengths. The procedure was as follows: One vagus nerve was cut and the normal effect of stimulation established; then the corresponding ganglion was removed and the nerve again stimulated, and finally, the other ganglion was removed and the vagus stimulated. I shall state the results very briefly.

Respiration.—Stimulation of the vagus nerve after removal of the corresponding ganglion, or of both ganglia, gave results similar to those obtained before the removal, that is, active respiration, passive respiration (inhibition of the diaphragmatic movements), acceleration of the respiration, or a tetanic standstill of the diaphragm in an inspiratory state, according to the strength of the stimulus used and the individuality of the animal. *Bloodpressure.*—Stimulation of the vagus nerve gave a definite rise, and stimulation of the depressor nerve gave a fall. Rise and fall after removal of the ganglia were—at least—as good as before the removal. *Deglutition.*—When the animal was not too deeply narcotized, stimulation of the vagus trunk produced irregularly one deglutition or more, and stimulation of the central end of the superior laryngeal nerve produced regularly a series of swallows. *Sensation.*—Finally, it may be added that when the animal was nearly out of ether, stimulation with a strong current brought out signs of sensation, which gave the signal for an increase of the anesthesia. *These experiments show unmistakably that the afferent nerve fibres within the vagus were in no visible way affected by the act of removing the ganglia.*

The Centrifugal Fibres.—The efferent nerve fibres of the vagus, which concern us here, are those which innervate the muscles of the larynx, the pharyngeal muscles and the muscular fibres of the esophagus. The laryngeal innervation after removal of the ganglia has not yet been studied. However, clear evidence was obtained that the motor nerve fibres attending the pharynx and the esophagus were functioning in a normal way. *Under moderate anesthesia the inferior pharyngeal muscles were seen to contract with the rise of the*

larynx, which accompanies the initial stage of deglutition, and the rise was followed by a normal peristaltic wave in the esophagus.

The condition of the esophagus at the autopsy is another evidence that the vagus was not affected by the procedure of the removing of both ganglia. In the autopsies made on the twenty-five rabbits which died after the removal of both ganglia, the esophagus was found empty and contracted in fourteen cases; in the other cases the condition of the esophagus was not noted. In other words, in all the cases in which the esophagus was examined, it was found that after removal of the ganglia this tube was empty and contracted. On the other hand, in a series of eighteen rabbits in which both vagi were cut and in which nearly all animals died in less than twenty-four hours, at the autopsy the esophagus was found distended and filled with food in its entire length in thirteen animals; in the remaining five the esophagus was either only partly filled or entirely empty but was not exactly contracted. I wish to point out here that the upper part of the esophagus is partly innervated by pharyngeal nerves which remain intact when both vagi are cut in the neck. Moreover, food may be driven out by the rigor of the esophagus which, after the rigor passes away, may be relaxed again. At any rate, *it is a noteworthy fact that after the removal of the ganglia the esophagus was found empty and contracted in all cases in which the state of that tube was looked for, while in thirteen out of eighteen animals in which both vagi were cut, the entire esophagus was distended and full of food, a fact which speaks for the contention that the vagus nerves have not been affected by the removal of the ganglia.*

From the foregoing evidences, I am inclined to conclude *that the death of the animals in which both ganglia were removed was due to the removal of these organs—if I may call them so—and not due to the procedure of the operation. In other words, the superior cervical ganglia contain a principle which is essential for the maintenance of life.*

As to the nature of the pulmonary lesions which were found after death following the removal of both ganglia, I shall only say that nearly in all instances there was a bronchopneumonia which was mostly located in the upper and middle lobes, and a pulmonary edema located mostly in the lower lobes of the lungs. In a few cases there were large and small abscesses in some parts of the lungs, and in some

lungs there were plugs in the small bronchi which were apparently connected with the abscesses and which consisted of fibrin, pus and foreign material. In a few instances there were also serous or purulent pleurisies. I shall not attempt to enter into further details. Furthermore, I shall not discuss the question whether there is any difference between the pulmonary lesions observed after the removal of both ganglia and the lesions found in the lungs after section of both vagi. This I can the more afford to do, since the centuries' old question as to the nature of the pulmonary lesions after section of both vagi was recently opened up again by the important researches of Schafer,[3] and the entire question will surely have to be a subject of further investigations.

As to the nature of the mechanism by which the ganglia may control life, I shall, for the present, offer only a hypothesis. At the beginning of this communication, I called attention to the fibres from the superior cervical ganglia which enter the "brain," and put forward the question, whether some of these fibres may not terminate in a vital part of the medulla oblongata. The hypothesis answers this question in the affirmative, namely, that the fibres reach the respiratory centre in which they exert a controlling influence upon the coördinate antagonistic activities of the laryngeal muscles in the function of respiration. As we know, the glottis becomes wider during inspiration and narrower during expiration and is otherwise changing its configuration in the different respiratory phases. The larynx is provided with extrinsic and intrinsic muscles which act alternately as adductors and abductors and are innervated by different fibres running in the trunks of the laryngeal nerves. According to the law which I termed *Antagonistic Innervation* or the law of *Reciprocal Innervation* (Sherrington), the adductor muscles of the larynx become inhibited during the contraction of the abductors, and the latter become inhibited during the contraction of the adductors. Such complicated continuous muscular activities suggest the control of a centre. This central control is maintained in a state of tonus. The hypothesis assumes that the tonus of the central laryngeal control is maintained by impulses coming continuously from the superior cervical ganglia. Some time after the removal of these ganglia the respiratory laryngeal centre loses its tonus and its coördinating activity upon the laryngeal muscles

[3] Schafer, E Sharpey, *Quart. J. Exper. Physiol.*, 12, 1919 (231–301).

during respiration. The loss of coördination gives rise to results similar in some way to those consequent upon cutting of the vagus nerves. The difference between the two conditions is, that in cutting the vagus nerves, the *peripheral (motor) innervation of all the muscles* are simultaneously abolished, while after removal of the ganglia *only the central control* is eliminated. In this case impulses may still reach the laryngeal muscles from the respiratory centre, but they will now contract in an incoördinate and harmful manner. Future experiments may reveal that the behavior and appearance of the glottis after removal of the ganglia differ from its behavior and appearance after cutting of the vagi.

This supposed reflex action of the ganglia upon the control of the antagonistic contraction of the laryngeal muscles is somewhat parallel to the action of the same ganglia upon the peripheral organs, for instance, upon the iris. In this instance it controls the width of the pupil by causing either a contraction of the dilator muscle simultaneously with a relaxation of the sphincter pupilae, or, it causes a constriction of the sphincter with a simultaneous relaxation of the dilator.

As to the nature of the origin of the impulses, I compared above the ganglia with the glands of internal secretion. I append here a very brief report of a few experiments, the making of which was stimulated by the mentioned comparison. In four rabbits both superior cervical ganglia were torn away from their upper connections, but were left connected with the sympathetic nerves. In addition, two ganglia from another rabbit were placed deep in the wound before closing it. *All four animals survived many weeks* and were killed later by chloroform; the lungs were found normal. A fifth rabbit was killed six days after the operation in an experiment in which by accident only one foreign ganglion was added. At the autopsy a small abscess was found in the right middle lobe. Probably, the animal would have recovered. These few experiments do not permit any definite conclusion; but the findings are suggestive. If further experimentation should give similar results, the question would be obvious: *whether other sympathetic ganglia also possess some sort of an internal secretion.*

SYNTHESES IN THE CINCHONA SERIES.

IV.[1] Nitro- and Amino-Derivatives of the Dihydro Alkaloids.

By WALTER A. JACOBS and MICHAEL HEIDELBERGER.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

In the preparation of 5-nitro-dihydro-quinine
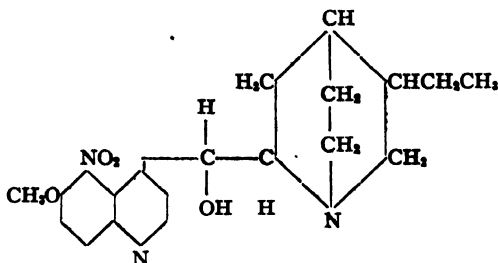
and 5-amino-dihydro-quinine according to German patent No. 283,537, difficulties were encountered which had to be overcome before the amino compound could be obtained readily enough to serve as a starting point for certain synthetic work which we had contemplated. In the first place, nitro-dihydro-quinine is described as resulting from the nitration of dihydro-quinine sulfate by the use of a nitric-sulfuric acid mixture, with no reference to the exact proportions employed. A study of the reaction showed that the formation of nitro-dihydro-quinine was complicated by the production of a nitro-dihydro-quinine sulfonic acid. Although this did not occur when the sulfate was added to a mixture of equal volumes of nitric and sulfuric acids, the tendency toward sulfonation increased as the proportion of sulfuric acid to nitric acid was increased, with consequent diminution of the yield of nitro-dihydro-quinine. It was then found that a quantitative conversion of dihydro-quinine into the nitro

[1] Cf. This Journal, 41, 817, 2090, 2131 (1919).

base could easily be realized with fuming nitric acid, and this was finally adopted as the most satisfactory reagent since it is less viscous and dissolves the alkaloid more rapidly than the mixture of equal parts of sulfuric and nitric acids, and also yields a less highly colored product.

Experiments with nitro-dihydro-quinine sulfonic acid explained its occurrence, since it was shown that this substance is formed both by nitration of dihydro-quinine sulfonic acid and by sulfonation of nitro-dihydro-quinine. In the latter case, it is sufficient to allow a solution of the nitro base in conc. sulfuric acid to stand at room temperature, a result in no way surprising, since the dihydro alkaloids have been found to yield sulfonic acids readily under the same conditions.[2] Since the nitro acid may be obtained from dihydro-quinine sulfonic acid, the sulfonic acid group occupies the same position in both of these acids, but according to Schmid's[3] experiments with cinchotine sulfonic acid, it is not situated in the quinoline nucleus. The lability of the sulfonic acid group toward boiling hydrochloric acid and its stability in boiling alkaline solution as demonstrated by Schmid for dihydro-quinine sulfonic acid, are properties retained by the nitro acid.

By following the method for the reduction of nitro-dihydro-quinine given in the patent we obtained but poor yields of the amino alkaloid, and it was necessary to modify the method considerably before a smooth conversion of the nitro alkaloid into the amino compound was afforded. In extending the work, the methods outlined were successfully applied to ethyl-dihydro-cupreine (optochin), dihydro-quinidine, ethyl-dihydro-cupreidine,[4] and dihydro-quinane,[5] resulting in the synthesis of the corresponding nitro and amino derivatives. Like 5-amino-quinoline, the amino alkaloids form orange-red, non-fluorescent solutions in dilute acids and in addition give the thalleo-quinine reaction. This reaction is not given by the nitro compounds.

[2] Hesse, *Ann.*, **241**, 283 (1887); Skraup, *Monatsh.*, **18**, 414 (1897).

[3] Schmid, *ibid.*, **22**, 803 (1901).

[4] THIS JOURNAL, **41**, 830 (1919).

[5] See following paper.

EXPERIMENTAL PART.

5-Nitro-dihydro-quinine.—Although this substance was prepared by the use of a nitration mixture consisting of equal parts of conc. nitric and sulfuric acids as in the preparation of dinitro-quinine by Rennie,[6] fuming nitric acid was found to give the most satisfactory results with dihydro-quinine and was, therefore, employed in the other cases described below.

Fifty g. of anhydrous dihydro-quinine sulfate were added in small portions to 200 cc. of fuming nitric acid (sp. gr. 1.52), with turbining, and keeping the temperature at 0°. The salt dissolved rapidly, and after addition of the full amount the clear solution was allowed to stand for 15 minutes at 0° and then poured on to ice. After dilution to about 4 liters the solution was treated with 25% aqueous sodium hydroxide until most of the nitric acid was neutralized and the alkaloid still remained dissolved as the nitrate. The solution was then vigorously stirred and the base quickly precipitated by the rapid addition of ammonia in excess. If the precipitation is too slow, or if the solution is not sufficiently dilute, a gummy mass of what is presumably the mono nitrate separates and is difficult to transform into an easily filterable form. The pale yellow, partially crystalline precipitate is filtered off, washed well with water, and dissolved in dil. hydrochloric acid. After adding an equal volume of alcohol, warming, and making alkaline with ammonia, the nitro-dihydro-quinine separates rapidly as lustrous yellow plates. After washing with 50% alcohol the yield is 90%.

For final purification the base was dissolved in 50% alcohol by the addition of sufficient acetic acid, and, after warming again, precipitated with ammonia, forming lustrous, pale yellow, hexagonal scales which decompose at 220–2° with preliminary darkening and softening, and not at 209–12° as given in the patent. The nitro compound is appreciably soluble in methyl or ethyl alcohol, acetone or benzene, more readily so in the hot solvents. It is easily soluble in chloroform and dil. acids and very sparingly in ether. It does not give the thal-'

[6] Rennie, *J. Chem. Soc.*, **39**, 470 (1881).

leoquinine test, and on exposure to sunlight turns a purplish brown. $[\alpha]_D^{25} = -200.0°$ in chloroform; $c = 1.350$.

Subs., 0.1293: 12.91 cc. N (25.0°, 760 mm.).
Calc. for $C_{20}H_{25}O_4N_3$: N, 11.32.   Found: 11.42.

5-Nitro-dihydro-quinine Sulfonic Acid.—(A). From nitro-dihydro-quinine. This substance was obtained as the main product of the reaction when the nitration of dihydro-quinine was attempted with a mixture of sulfuric acid and the theoretical amount of conc. nitric acid. It was then found that the compound results either from the nitration of dihydro-quinine sulfonic acid, or by allowing a solution of nitro-dihydro-quinine in conc. sulfuric acid to stand at ordinary temperature, just as in the formation of dihydro-quinine sulfonic acid.

Five g. of nitro-dihydro-quinine were dissolved in 25 cc. of conc. sulfuric acid and allowed to stand for 24 hours. After pouring on to ice and diluting, the greenish yellow solution was partly neutralized with sodium hydroxide and finally made neutral to Congo red with sodium acetate solution. On rubbing the acid separates as yellow needles which were washed with water. It was recrystallized by dissolving in dil. alkali, warming, and reacidifying with acetic acid, a transitory turbidity appearing at the neutral point. The acid separates as flat, glistening, yellow needles which rapidly turn green on exposure to sunlight. It chars when heated above 260° but does not melt below 285°, and is practically insoluble in the usual solvents except boiling 50% alcohol. It dissolves in dil. hydrochloric and nitric acids but less readily in dilute sulfuric acid. The addition of strong sodium hydroxide or sodium chloride solution to the solution of the acid in dil. sodium hydroxide salts out the sodium salt as a gummy precipitate. Like dihydro-quinine sulfonic acid, the nitro acid is relatively stable in alkaline solution but on boiling with 20% hydrochloric acid the sulfonic group is quantitatively eliminated with the formation of nitro-dihydro-quinine. This property was, therefore, employed for the sulfur determination. The position occupied by the sulfonic acid group was not determined, although it was shown to be the same as that occupied by the sulfo group in dihydro-quinine sulfonic acid, since this yielded the same nitro compound. $[\alpha]_D^{25} = -133.0°$ in 0.5 N NaOH; $c = 1.000$.

Subs., 0.1240: 10.6 cc. N (28.0°, 759 mm.).   Subs., 0.1509: BaSO₄, 0.0751.
Calc. for C₂₀H₂₅O₇N₃S: N, 9.31; S, 7.10.   Found: N, 9.69; S, 6.84.

(*B*). From dihydro-quinine sulfonic acid. Dihydro-quinine sul-
fonic acid was obtained by allowing a solution of 10 g. of anhydrous
dihydro-quinine sulfate in 50 cc. of conc. sulfuric acid to stand for
24 hours. After dilution with water and partial neutralization with
sodium hydroxide solution, the mixture was neutralized to Congo
red with sodium acetate, causing the separation of 8.7 g. of the sul-
fonic acid as lustrous, colorless rhombs, often grouped as serrated
aggregates. This was added in small portions to 30 cc. of fuming
nitric acid (sp. gr. 1.52) kept at 0°. After 15 minutes' standing at
0°, the mixture was poured on to ice and the acid isolated as previously
described. After recrystallization 8.7 g. of slightly greenish yellow,
flat, glistening needles were obtained, identical in all respects with the
acid obtained from nitro-dihydro-quinine. $[\alpha]_D^{27} = -129.6°$ in 0.5
N NaOH; $c = 1.012$.

Subs., 0.1201: 10.0 cc. N (23.5°, 759 mm.).   Subs., 0.2005: BaSO₄, 0.1052
Calc. for C₂₀H₂₅O₇N₃S: N, 9.31; S, 7.10.   Found: N, 9.58; S, 7.21.

*Hydrolysis of Nitro-dihydro-quinine Sulfonic Acid to Nitro-dihydro-
quinine.*—2 g. of the sulfonic acid dissolved in 25 cc. of 1:1 hydro-
chloric acid were boiled for one hour. An equal volume of alcohol
was added and the solution made alkaline with ammonia. The
crystalline precipitate was recrystallized as previously described,
forming lustrous, pale yellow, hexagonal scales which decomposed
at 220–2° and proved identical with the 5-nitro-dihydro-quinine
prepared by the direct method.

Subs., 0.1312: 13.2 cc. N (24.0°, 758 mm.).
Calc. for N, 11.32.   Found: 11.54.

5-Amino-dihydro-quinine.—On repeating the directions given in
German patent 283,537 for the reduction of nitro-dihydro-quinine, it
was found that when the reaction mixture was made alkaline before
extracting the amino compound with ether, it rapidly turned deep
green owing to oxidation, showing that decomposition had occurred.
In consequence, the yield of amino-dihydro-quinine was poor, only
5.8 g. being recovered from 18 g. of the nitro compound. A number

of experiments finally led to the following satisfactory method, which was then applied to the reduction of the other nitro cinchona derivatives described below.

18.5 g. of nitro-dihydro-quinine were dissolved in 185 cc. of conc. hydrochloric acid with chilling and stirring, the alkaloid being slowly added in order to diminish the tendency to form a gum.   After chilling the solution to 0°, 45 g. of stannous chloride were added.   On removing the beaker from the freezing mixture and stirring, the temperature rose as the reaction proceeded, but was not allowed to exceed 35°.   A thick, partially crystalline, yellow paste of the tin salt separated, and after standing for 15 minutes, the mass was dissolved in water and ice added.   The deep orange-red solution was then treated with an excess of 25% sodium hydroxide solution, no trace of green coloration being evident.   The amorphous amino compound was shaken out with about one liter of ether, the yellow solution depositing most of the substance as yellow needles on standing.   On concentration the mother liquor yielded the remainder, the total being 15 g. Recrystallized from hot benzene, in which it is fairly easily soluble at the boiling point, it separates as rosets of thickly matted, minute, yellow, microscopic needles which melt at 220–1° with slight preliminary softening.   Giemsa and Halberkann[7] give 217–8° as the melting point, while in German patent 283,537 it is given as 208–12°.   The base is very readily soluble in chloroform, quite easily in methyl or ethyl alcohol, less so in acetone, and very sparingly in ether or cold benzene.   It gradually turns a brownish purple on exposure to sunlight.   It dissolves in dilute acids with an orange-red color and gives the thalleoquinine reaction, and, as we shall describe more fully in a subsequent communication, it couples smoothly with diazo compounds to form well defined amino-azo dyes. $[\alpha]_D^{21} = -17.7°$ in absolute   alcohol; $c = 1.020$.

Subs., 0.1187: 12.85 cc. N (20.0°, 749 mm.).
Calc. for $C_{20}H_{27}O_2N_3$: N, 12.31.   Found: 12.44.

5-Nitro-ethyl-dihydro-cupreine (*5-Nitro-optochin*).—20 g. of anhydrous ethyl-dihydro-cupreine sulfate (from a solution of the chloride with ammonium sulfate) were nitrated as in the case of dihydro-qui-

[7] *Ber.*, **52**, 922 (1919).

nine sulfate.   The base, precipitated from the diluted reaction mixture·
with ammonia, first separated amorphous and then partly crystallized.
It was recrystallized by dissolving in 50% alcohol with the aid of
acetic acid and reprecipitated by the addition of ammonia to the
warm solution.   The base separated in excellent yield as lustrous,
pale yellow, hexagonal platelets which melted and decomposed at
225–6° with preliminary darkening and softening after another
recrystallization from toluene.   It is rather difficultly soluble in cold
alcohol or acetone but readily on boiling, and is more easily soluble
in cold methyl alcohol or chloroform.   A concentrated neutralized
solution of the nitro alkaloid in hydrochloric acid deposits pale
yellow, delicate needles of the hydrochloride. $[\alpha]_D^{22.5} = -198.2°$ in
chloroform; $c = 0.515$.

> Subs., 0.1267: 12.15 cc. N (23.5°, 760 mm.).
> Calc. for $C_{21}H_{27}O_4N_3$: N, 10.91.   Found: 11.04.

5-Amino-ethyl-dihydro-cupreine (5-Amino-optochin).—18.5 g. of the
nitro compound, treated as in the case of nitro-dihydro-quinine,
yielded 14 g. of the amino alkaloid after recrystallization from dil.
alcohol with the aid of boneblack.   Recrystallized from 85% alcohol,
the base separates as minute, lemon-yellow platelets which melt with
slow decomposition at 214–5° with slight preliminary softening and
darkening.   It is very readily soluble in chloroform, quite easily in
methyl and ethyl alcohols and less readily in acetone, forming yellow
solutions.   It dissolves in hot benzene, separating in gelatinous form
on cooling.   The solution in dil. hydrochloric acid is orange in color,
and gives the thalleoquinine reaction. $[\alpha]_D^{24.5}$ is $-15.9°$ in absolute
alcohol, $c = 1.034$, while Giemsa and Halberkann[8] give $[\alpha]_D^{20} =$
$-13.2°$ and the melting point as 211–2°.

> Subs., 0.1225: 12.55 cc. N (23.0°, 770 mm.).
> Calc. for $C_{21}H_{29}O_2N_3$: N, 11.83.   Found: 11.97.

5a-Nitro-dihydro-quinidine.—46.5 g. of anhydrous dihydro-quinidine
sulfate were nitrated as in the previous cases, the final precipitation
of the free base being carried out rapidly with ammonia from the

[8] *Loc. cit.*, pp. 922–3.

highly diluted reaction mixture. If the precipitation is too slow, or if the solution is too concentrated the substance separates largely as a gum consisting of the nitrate of the base. It is advisable even when precipitation is properly accomplished to grind the filtered and washed base with dil. ammonia and to filter and wash again. The yield of amorphous base was 43 g. and was sufficiently pure for reduction to the amino alkaloid.

On dissolving the dried base in hot ethyl acetate and allowing to cool rapidly it separates as a jelly, but when the concentrated solution is kept warm and rubbed, the nitro compound separates slowly as yellow rhombs. The same phenomena occur in 50% alcohol, since crystallization is slow, and in order to avoid contamination with amorphous material the solutions must be kept warm. The crystalline base is readily soluble in alcohol, chloroform, acetone, and ethyl acetate, less easily in benzene and very sparingly in ether. It melts and decomposes at about 208–9° with preliminary darkening and softening, and does not give the thalleoquinine test. $[\alpha]_D^{23} = + 326.5°$ in absolute alcohol; $c = 0.668$.

Subs., 0.1082: CO_2, 0.2579; H_2O, 0.0647.   Subs., 0.1307: N, 13.2 cc. (23.0°, 765 mm.).

Calc. for $C_{20}H_{25}O_4N_3$: C, 64.65; H, 6.79; N, 11.32.   Found: C, 65.00; H, 6.69; N, 11.72.

The Nitrate.—This salt was obtained in one experiment in which the insufficiently diluted reaction mixture from the nitration of dihydroquinidine sulfate had been precipitated with ammonia, the precipitate consisting mostly of the nitrate. Although flocculent at first, it sintered to a gum which prevented filtration, but was collected and dissolved in 95% alcohol, the salt separating as pale yellow rhombs, which were again crystallized from alcohol. The air-dry salt contains solvent approximately equivalent to 2 molecules of water of crystallization, and like the anhydrous salt intumesces at 142–5°. It is slowly but fairly readily soluble in water with a bright yellow color and dissolves readily in methyl and ethyl alcohols, acetone and chloroform, and only sparingly in hot benzene. $[\alpha]_D^{22}$ of the anhydrous salt is $+ 232.8°$ in water; $c = 0.896$.

Air-dry: Subs., 0.6727: loss, 0.0570, *in vacuo* at 80° over H_2SO_4.
Calc. for $C_{20}H_{25}O_4N_3.HNO_3.2H_2O$: H_2O, 7.66.   Found: 8.47.
Anhydrous: Subs., 0.1112: 12.0 cc. N (22.0°, 763 mm.).
Calc. for $C_{20}H_{25}O_4N_3.HNO_3$: N, 12.90.   Found: 12.54.

5-Amino-dihydro-quinidine.—18.5 g. of the dried, amorphous nitro alkaloid yielded 11.5 g. of the amino compound. The substance separates slowly from the ethereal extract as olive-yellow rhombs. Recrystallized from 95% alcohol, it forms minute, rhombic platelets which, when rapidly heated to 235°, then slowly, darken and decompose at 238–42°. The base is somewhat less soluble in cold alcohol than in methyl alcohol, forming yellow solutions, and is more easily soluble in hot chloroform than in hot acetone. It gives the thalleoquinine reaction and dissolves in dil. hydrochloric acid with an orange-red color which appears orange-brown in thin layers. $[\alpha]_D^{20}$ is + 115.5° in absolute alcohol; $c = 0.870$.

Subs., 0.1230: N, 13.05 cc. (24.5°, 768 mm.).
Calc. for $C_{20}H_{27}O_2N_3$: N, 12.31.   Found: 12.30.

5-Nitro-ethyl-dihydro-cupreidine.—5 g. of ethyl-dihydro-cupreidine[9] were slowly added to 25 cc. of fuming nitric acid (sp. gr. 1.52), keeping the temperature below 0°. Five cc. of conc. sulfuric acid were then added, and after standing for 20 minutes in the cold the mixture was poured into 1500 cc. of water. The nitro base was precipitated with ammonia and shaken out with ether, which, on concentration, yielded 4–5 g. of the crystalline nitro compound. Recrystallized as in previous examples from 50% alcohol, it separates as rosets and plumes of minute, narrow, pale yellow, glistening platelets which melt and decompose at 220–1° with preliminary darkening and softening. It is quite soluble in alcohol, acetone, benzene and ether, and very readily so in chloroform. $[\alpha]_D^{22}$ is + 322.4° in absolute alcohol; $c = 0.951$.

Subs., 0.1082: CO_2, 0.2593; H_2O, 0.0688.   Subs., 0.1194: 11.3 cc. N (22.0°, 758 mm.).
Calc. for $C_{21}H_{27}O_4N_3$: C, 65.41; H, 7.07; N, 10.91.   Found: C, 65.36; H, 7.12; N, 10.92.

[9] THIS JOURNAL, **41**, 830 (1919).

5-Amino-ethyl-dihydro-cupreidine.—The nitro compound was reduced as in previous examples, but an entirely pure product was not obtained. The ethereal solution of the amino alkaloid left a crystalline residue on evaporation, which was recrystallized first from 85% alcohol, then from not too little toluene, separating on cooling as rosets of minute, lemon-yellow needles which melt at 216–7° with slight preliminary darkening and show $[\alpha]_D^{26.5} = -69.4°$ in dry chloroform; $c = 1.017$, $[\alpha]_D^{26.5} = +91.6°$ in absolute alcohol, $c = 0.524$. The base is rather difficultly soluble in alcohol, more easily in methyl alcohol, and fairly readily in dry chloroform, especially on warming. It is sparingly soluble in cold toluene, readily at the boiling point, separating in gelatinous form on cooling unless the solution is seeded and permitted to crystallize while still warm. It gives the thalleoquinine reaction.

Subs., 0.1166: CO_2, 0.3007: H_2O, 0.0834. Subs., 0.1309: 14.0 cc. N (23.5°, 770 mm.).

Calc. for $C_{21}H_{29}O_2N_3$: C, 70.93; H, 8.23; N, 11.83. Found: C, 70.33; H, 8.00; N, 12.48.

5-Nitro-dihydro-quinane.—Dihydro-quinane trihydrate[10] was nitrated in the same way as was ethyl-dihydro-cupreidine. The partially crystalline nitro compound was shaken out with ether, which was dried and the solution then concentrated. The residue was taken up in 85% alcohol and diluted with water until the initial turbidity just redissolved. On rubbing and cautiously adding further small amounts of water, the hydrated nitro compound separated in a yield equal to that of the dihydro-quinane used. Recrystallized from 50% alcohol it forms radiating masses of pale yellow, delicate needles which retain 3.5 molecules of water of crystallization when dried to constant weight in a desiccator over water. When dried in the air, water is lost and the substance sinters to a gum. The hydrate begins to soften above 60° and melts to a turbid liquid at 67.5–9°. It dissolves readily in alcohol, acetone, or chloroform, and rather less easily in ether. It does not give the thalleoquinine test. $[\alpha]_D^{22}$ is + 80.2° in 95% alcohol; $c = 1.059$.

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[10] See following paper.

Air-dry: Subs., 0.4842: loss, 0.0717 *in vacuo* at 80° over H_2SO_4.   Subs., 0.1218: 10.8 cc. N (23.5°, 757 mm.).

Calc. for $C_{20}H_{25}O_3N_3.3.5\ H_2O$: H_2O, 15.07; N, 10.04.   Found: H_2O, 14.80; N, 10.17.

An attempt to reduce this substance failed owing to the insolubility of its tin double salt.

SUMMARY.

Improved methods are given for the preparation of 5-nitro- and 5-amino-dihydro-quinine, and it is shown that under the usual conditions of nitration with nitric and sulfuric acids sulfonation also occurs and 5-nitro-dihydro-quinine sulfonic acid is formed as a by-product. The extension of the methods of nitration and reduction to ethyl-dihydro-cupreine (optochin), dihydro-quinidine and ethyl-dihydro-cupreidine led to the formation of the nitro and amino derivatives of these alkaloids, and descriptions of these new substances are given.   5-Nitro-dihydro-quinane (see next paper) is also described.

SYNTHESES IN THE CINCHONA SERIES.

V. Dihydro-Desoxy-Quinine and Dihydro-Desoxy-Quinidine and Their Derivatives.

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(Received May 4, 1920.)

As a part of our studies on the cinchona alkaloids it became of interest to examine the biological properties of the so-called "desoxy" compounds, in which the secondary alcoholic group of the parent bases
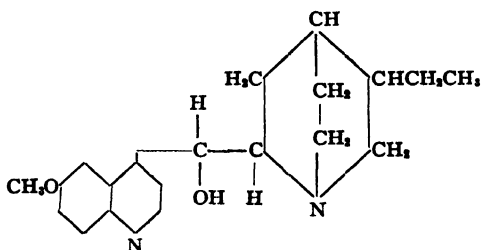
has been replaced by the hydrogen atom.
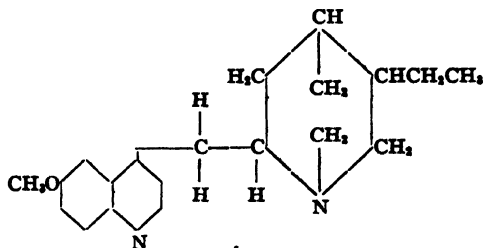
While several of these modified alkaloids have been prepared and studied chemically by Koenigs[1] and Rabe[2] and their co-workers, we are unaware of any previous publications on the desoxy compounds of dihydro-quinine and dihydro-quinidine or their immediate derivatives.

In reporting this work we have adopted a slight modification of the terminology hitherto used in this series, a modification which we believe will avoid the use of unwieldy combinations and take care of any further expansion in this field. Using as a basis Koenigs' adoption of the term "quinene" for

CH
H₃C
CH₂
CHCH: CH₂
H
C=C
CH₂
CH₃
CH₃O
N
N
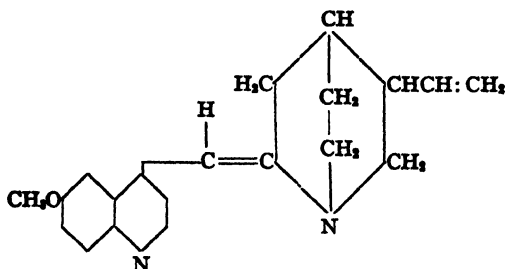
and Rabe's use of the term "quininone" for

CH
H₃C
CH₂
CHCH: CH₂
C—C
CH₂
CH₃
CH₃O
O   H
N
N
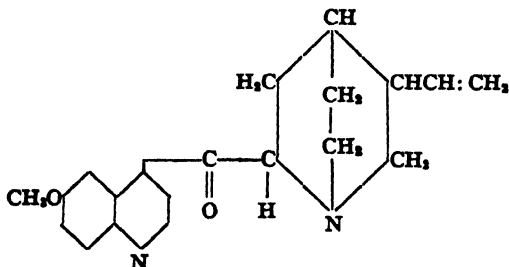
we have employed the suffix "-ane" to indicate "desoxy-." "Desoxyquinine" and its stereoisomer would then become "quinane" and "quinidane," while the awkward dihydro-desoxy-quinine and dihydro-desoxy-quinidine become dihydro-quinane and dihydro-quinidane. Similarly, the terms "cinchonane" and "dihydro-cupreane" seem preferable to desoxy-cinchonine and dihydro-desoxy-cupreine.

[1] Koenigs, *Ber.*, **28**, 3147 (1895); **29**, 372 (1896).

[2] Rabe, *Ann.*, **373**, 107 (1910).

Adopting this terminology then, the following series was studied, dihydro-quinine → chloro-dihydro-quinine → dihydro-quinane → dihydro-cupreane → ethyl-dihydro-cupreane; or, graphically.
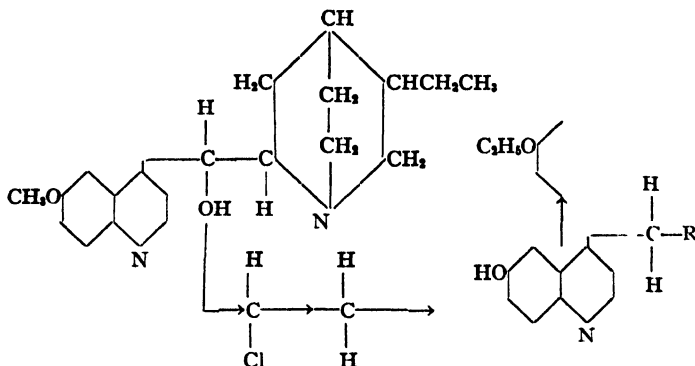
While it would no doubt have been possible to complete the corresponding series starting with the dextrorotatory dihydro-quinidine, we have carried our chemical studies with the entire group only as far as was necessary to establish the fact that these compounds are less bactericidal for the pneumococcus than the corresponding parent alkaloids.

The methods used for the preparation of the chloro- compounds and the "quinanes" were essentially those employed by Koenigs and Rabe for the corresponding non-hydrogenated derivatives, with the exception that the "quinanes" were isolated directly from the reaction mixture as the picrates. The demethylation of dihydro-quinane and dihydro-quinidane was accomplished by boiling with hydrobromic acid (sp. gr. 1.49).[3]

With the exception of ethyl-dihydro-cupreane all of the new bases crystallized fairly readily, and were further characterized by the preparation of crystalline mono-acid and di-acid salts. Dihydro-quinane and its stereoisomer could be crystallized, however, only in the form of hydrates, a property already noted by Koenigs in the case of quinane and quinidane. The 2 phenolic alkaloids, dihydro-cupreane and dihydro-cupreidane showed themselves entirely analogous in their properties to dihydro-cupreine and dihydro-cupreidine.

[3] Cf. THIS JOURNAL, 41, 821 (1919).

A. Derivatives of Dihydro-quinine.

Chloro-dihydro-quinine.—133 g. of anhydrous dihydro-quinine dihydrochloride were worked up into a smooth, thin paste with dry chloroform and poured into a suspension of 200 g. of phosphorus pentachloride in dry chloroform, checking the first evolution of heat by immersion in a freezing mixture. A voluminous, pale yellow precipitate formed, and there was a steady evolution of hydrogen chloride as the mixture came up to room temperature. During the next 4 or 5 days the mixture was heated in a water bath at 45–55° with frequent shaking, and was then cooled and treated with ice until no further evolution of heat was apparent. The base was precipitated from the aqueous layer by means of sodium hydroxide, taken up with ether, dried over sodium sulfate, and the solvent distilled off. The crystalline residue was taken up in boiling benzene and the solution treated with several volumes of ligroin (b. p. 80–90°), the chloro-dihydro-quinine separating on seeding as cream-colored rhombs. The yield was 67 g. Recrystallized first from dil. alcohol, then from ligroin, it forms large rhombic aggregates which melt slowly at 143–4° with slight preliminary softening. The substance dissolves readily in alcohol, acetone, chloroform, or benzene, less readily in ether, and is very sparingly soluble in cold ligroin but fairly readily so at the boiling point. It gives a pale yellow, non-fluorescent solution in dil. sulfuric acid. $[\alpha]_D^{21.5}$ is $+ 42.1°$ in absolute alcohol; $c = 1.365$.

Subs., 0.1869: 13.4 cc. N (22.0°, 757 mm.).
Subs., 0.1534: (Carius) AgCl, 0.0635.
Calc. for $C_{20}H_{25}ON_2Cl$: N, 8.13; Cl, 10.28. Found: N, 8.27; Cl, 10.24.

The Hydrochloride.—A solution of the base in dil. hydrochloric acid was neutralized to litmus and treated with sodium chloride until the initial turbidity just redissolved on shaking. The salt gradually separated, and was recrystallized from 50% alcohol, separating on seeding and letting stand in the ice-box as aggregates of narrow plates which are anhydrous and dissolve rather sparingly in cold water or alcohol, more easily on heating. The aqueous solution is colorless, but turns a pale yellow when excess acid is added. The hydrochloride begins to

darken and decomposes at 230°, melting with decomposition at 232–3°. $[\alpha]_D^{20.5}$ is − 2.9° in water; $c = 1.197$.

Subs., 0.1387: 8.8 cc. N (20.0°, 760 mm.).
Subs., 0.1700: 8.89 cc. AgNO₃ sol. (1 cc. = 0.001794 g. Cl).
Calc. for $C_{20}H_{25}ON_2Cl.HCl$: N, 7.35; Cl⁻, 9.31. Found: N, 7.40; Cl⁻, 9.38.

Dihydro - quinane (*Dihydro - desoxy - quinine.*) — Chloro-dihydro-quinine was reduced with iron filings and dil. sulfuric acid according to the method of Rabe *et al.*[4] for the formation of similar desoxy compounds. Instead of extracting the base from the dried precipitate of iron hydroxides, we found it less time-consuming to filter, dilute the solution with an equal volume of water, and precipitate the alkaloid with an excess of 4% sodium picrate solution, making sure that the reaction remained acid to Congo red. The picrate, which filters readily, was washed with 1% sulfuric acid, ground to a thin paste with water, the mixture diluted, and then made strongly alkaline with sodium hydroxide. The base was shaken out with ether, the latter dried over sodium hydroxide, and concentrated. The residue, taken up in acetone and precipitated with water, crystallized on rubbing, owing to the formation of the *trihydrate*, the yield being 85% of the amount of chloro compound used. Recrystallized twice by dissolving in acetone, diluting with water to incipient turbidity, and rubbing, the trihydrate separated as silky needles which were brought to equilibrium for analysis in a desiccator containing only a beaker of water. When exposed to the air the crystals gradually lose their water, forming the oily, anhydrous base which could not be crystallized from dry solvents. The trihydrate dissolves readily in the usual organic solvents. A solution in dil. sulfuric acid shows a strong bluish fluorescence and is also relatively stable to permanganate. The base also gives a typical thalleoquinine test. $[\alpha]_D^{24}$ of the trihydrate is − 77.5° in absolute alcohol; $c = 1.244$.

Subs., 0.4048: loss, 0.0606 *in vacuo* at 100° over H_2SO_4.
Subs., 0.1142: CO₂, 0.2761; H, 0.0889.
Subs., 0.1181: 8.4 cc. N (23.0°, 764 mm.).
Subs., 0.1613: (Kjeldahl) 12.45 cc. 0.0714 N HCl.
Calc. for $C_{20}H_{26}ON_2.3H_2O$: C, 65.88; H, 8.86; N, 7.69; H_2O, 14.83. Found: C, 65.94; H, 8.71; N, 8.25, 7.72; H_2O, 14.97.

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[4] Ann., **373**, 107 (1910).

The Hydrochloride.—Dihydro-quinane was neutralized in alcoholic solution with hydrochloric acid, concentrated to dryness *in vacuo*, and the residue again taken down to dryness *in vacuo* after the addition of absolute alcohol. On adding dry acetone the salt soon crystallized as delicate needles which are anhydrous when air dried and melt at 179–80°. $[\alpha]_D^{22.5}$ is $-6.8°$ in water; $c = 1.034$. It dissolves readily in water, alcohol, chloroform, or methyl alcohol, and only difficultly in the cold in dry acetone or benzene, more easily on warming.

Subs., 0.1237: 9.0 cc. N (22.5°, 753 mm.).
Subs., 0.1293: 7.40 cc. AgNO₃ sol. (1 cc. = 0.00176 g. Cl).
Calc. for $C_{20}H_{26}ON_2.HCl$: N, 8.08; Cl, 10.22. Found: N, 8.33; Cl, 10.07.

The Dihydrochloride.—The base was treated with a slight excess of absolute alcoholic hydrochloric acid and then with dry acetone to initial turbidity. The salt crystallized on seeding with a test portion which was precipitated with dry acetone, decanted off, and let stand under dry acetone. Recrystallized by dissolving in absolute alcohol, adding a few drops of dry alcoholic hydrochloric acid, then about two volumes of dry acetone, followed by dry ether to incipient turbidity, the salt separated on seeding as faintly yellow, radiating masses of hair-like, felted needles which come to equilibrium in the air with solvent equivalent to 1.5 molecules of water of crystallization. When rapidly heated to 155°, then slowly, the salt softens to a jelly at 158–62°, gradually swelling and evolving gas, and becoming entirely fluid at about 210°. It dissolves readily in alcohol or chloroform, and very easily in water, yielding a non-fluorescent solution. When treated with a slight excess of hydrobromic acid (sp. gr. 1.49), then with acetone to incipient turbidity, a dihydrobromide separates on standing as long, delicate needles.

Air dry: Subs., 0.5360: loss, 0.0380 *in vacuo* at room temp. over H₂SO₄.
Calc. for $C_{20}H_{26}ON_2.2HCl.1.5$ H₂O; H₂O: 6.59. Found: 7.09.
Anhydrous: Subs., 0.1241: 8.05 cc. N (24.0°, 753 mm.).
Subs., 0.1232: 12.95 cc. AgNO₃ sol. (1 cc. = 0.00176 g. Cl).
Calc. for $C_{20}H_{26}ON_2.2HCl$: N, 7.31; Cl, 18.50. Found: N, 7.39; Cl, 18.50.

Dihydro-quinane Methiodide.—After several hours the solution of the components in dry acetone was diluted with water, the salt separating slowly on rubbing and letting the solution stand. Recrystal-

lized from 25% alcohol it separates on seeding as faintly yellow, glistening platelets and prisms which contain one molecule of water of crystallization and dissolve with difficulty in cold water and very easily on boiling. The anhydrous substance begins to sinter above 110°, forms a pale yellow jelly at 119–24°, and is completely fluid at about 145°. $[\alpha]_D^{21}$ is $-7.4°$ in absolute alcohol; $c = 1.145$. It dissolves very easily in dry chloroform, somewhat less readily in dry methyl or ethyl alcohol, and is almost insoluble in dry acetone.

Air dry: Subs., 0.7350: loss, 0.0286 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{21}H_{29}ON_2I.H_2O$: H_2O, 3.83. Found: 3.89.
Anhydrous: Subs., 0.1486: 8.1 cc. N (23.5°, 762 mm.).
Subs., 0.1104: 4.85 cc AgNO₃ sol. (1 cc. = 0.00642 g. I).
Calc. for $C_{21}H_{29}ON_2I$: N, 6.20; I, 28.07. Found: N, 6.29; I, 28.22.

Dihydro-cupreane (Dihydro-desoxy-cupreine).—25 g. of dihydro-quinane trihydrate were boiled with 100 cc. of hydrobromic acid (sp. gr. 1.49) until the temperature reached 125°, after which an air condenser was attached and the boiling continued for 3 hours.[5] The solution was then concentrated *in vacuo*, taken up in about 1.5 liters of water, and treated with 10% sodium hydroxide solution until all but a little gummy material had dissolved. This was redissolved in dil. hydrochloric acid and again made alkaline with sodium hydroxide, whereupon it all redissolved. The combined solutions were treated with boneblack and filtered to remove an almost negligible turbidity, and the filtrate treated with ammonium chloride solution. The resulting precipitate, and an additional portion obtained by shaking out the filtrate with chloroform, were dried and boiled with dry acetone, crystallization soon being complete. The yield was 15.8 g. Recrystallized from ethyl acetate, it separates on seeding as minute, delicate needles which melt at 191–1.5° to a yellow liquid filled with bubbles. It is very slightly soluble, with a faint yellow color, in boiling water, but dissolves readily in methyl or ethyl alcohol, or chloroform. It is sparingly soluble in cold benzene, but dissolves on warming. The base separates from methyl ethyl ketone as crusts of minute, thin, narrow platelets. $[\alpha]_D^{21}$ is $-77.1°$ in absolute alcohol; $c = 0.973$. Like dihydro-cupreine and dihydro-cup-

[5] *Loc. cit.*

reidine[6] it couples readily in alkaline solution with diazotized aromatic amines and does not give the thalleo-quinine reaction.

Subs., 0.1123: CO_2, 0.3165; H_2O, 0.0800.
Subs., 0.1175: 10.0 cc. N (28.0°, 748 mm.).
Calc. for $C_{19}H_{24}ON_2$: C, 76.97; H, 8.17; N, 9.46.   Found: C, 76.86; H, 7.97; N, 9.50.

The Hydrochloride.—Dihydro-cupreane was dissolved in a little absolute alcohol and neutralized to wet litmus with absolute alcoholic hydrochloric acid.   The salt soon crystallizes if water has been excluded.   Recrystallized from absolute alcohol, filtering the cooled solution from a slight turbidity, it separates on standing in the cold as thin, glistening platelets which melt at about 210° with slight decomposition. $[\alpha]_D^{23} = +1.5°$ in water; $c = 0.999$.   It dissolves readily in water with a greenish yellow color, the solution giving a brown color with ferric chloride and becoming almost colorless with an excess of hydrochloric acid.   It also dissolves readily in dry methyl alcohol without color, changing to yellow on adding water.   It is very difficultly soluble in boiling dry acetone or chloroform.

Subs., 0.1229: 9.1 cc. N (26.0°, 764 mm.).
Subs., 0.1227: 7.4 cc. $AgNO_3$ sol. (1 cc. = 0.00176 g. Cl).
Calc. for $C_{19}H_{24}ON_2.HCl$: N, 8.42; Cl, 10.66.   Found: N, 8.50; Cl, 10.62.

The Dihydrobromide.—In another preparation of dihydro-cupreane the hydrobromic acid solution was concentrated as far as possible *in vacuo*, finally in a boiling water bath, taken up in a small volume of absolute alcohol, and allowed to stand overnight in the cold.   The crude salt was recrystallized from 20% aqueous hydrobromic acid, after seeding with a test portion.   It forms delicate, radiating needles which contain one molecule of water of crystallization and dissolve readily in water.   The anhydrous salt slowly liquefies to a yellow fluid filled with bubbles at 225–7°.   It dissolves in dry methyl alcohol, less readily in absolute alcohol, and very difficultly in boiling dry acetone or chloroform.

[6] Cf. THIS JOURNAL, **41**, 827 (1919).

Air dry: Subs., 0.7651: loss, 0.0266 *in vacuo* at room temp. over H_2SO_4.
Calc. for $C_{19}H_{24}ON_2.2HBr.H_2O$: H_2O, 3.78.   Found: 3.48.
Subs., 0.1239: 10.85 cc. $AgNO_3$ sol. (1 cc. = 0.00396 g. Br).
Calc. for $C_{19}H_{24}ON_2.2HBr$: Br, 34.89.   Found: 34.70.

Dihydro-cupreane Methiodide.—The salt separated from chloroform solution. Recrystallized twice from absolute alcohol it forms thin, nacreous, faintly yellow platelets which dissolve in the cold in water, 50% alcohol, or methyl alcohol, and only sparingly in absolute alcohol but more easily on boiling. It is difficultly soluble in boiling dry acetone, chloroform, or benzene. $[\alpha]_D^{22}$ is $-$ 37.6° in 50% alcohol, $c = 1.050$. When rapidly heated to 160°, then slowly, it melts to a jelly at 160–5° and becomes completely fluid at 165–7°.

Subs., 0.1452: AgI, 0.0766.
Calc. for $C_{20}H_{27}ON_2I$: I, 28.97.   Found: 28.52.

Ethyl-dihydro-cupreane (*Dihydro-cupreane Ethyl Ether*) *Hydrochloride.*—Equimolecular amounts of dihydro-cupreane, 50% aqueous potassium hydroxide, and washed diethyl sulfate were allowed to stand in the cold in absolute alcoholic solution for 10 days.[7] The mixture was then treated with water and sodium hydroxide, shaken out with ether, and this dried over potassium hydroxide and concentrated. As the free base showed no tendency to crystallize it was neutralized to wet litmus with absolute alcoholic hydrochloric acid, treated with several volumes of dry ether, and seeded with crystals obtained from a similar test portion on long standing. The hydrochloride forms delicate needles which melt at 185–6° and dissolve readily in water, methyl or ethyl alcohol, acetone, or chloroform. The yield was poor. $[\alpha]_D^{23}$ is $-$ 9.0° in water; $c = 0.722$. It gives the thalleoquinine test, and fluoresces strongly in aqueous solution on addition of a drop of dil. sulfuric acid.

Subs., 0.1193: 8.4 cc. N (22.5°, 753 mm.).
Subs., 0.1158: 6.28 cc. $AgNO_3$ sol. (1 cc. = 0.00176 g. Cl).
Calc. for $C_{21}H_{28}ON_2.HCl$: N, 7.77; Cl, 9.83.   Found: N, 8.06; Cl, 9.55.

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[7] Cf. Ger. pat. 254,712; THIS JOURNAL, **41**, 830 (1919).

Bromo-dihydro-cupreine Dihydrobromide.—8 g. of chloro-dihydro-quinine were demethylated in the same way as was dihydro-quinane. During the concentration of the hydrobromic acid solution *in vacuo* the dihydrobromide crystallized and was filtered off after cooling and the concentration continued, an additional amount being obtained from the residue on taking up in water and seeding. The total yield was 10.6 g. Recrystallized from water containing a little hydrobromic acid it separates as glistening, cream-colored plates containing one molecule of water of crystallization. Analysis showed that the chlorine of the chloro-dihydro-quinine had been substituted by bromine. The salt dissolves in water with a pale yellow color, and after neutralization with sodium hydroxide gives a pale brown color with ferric chloride. $[\alpha]_D^{24}$ of the anhydrous salt is $-69.1°$ in water; $c = 1.005$. When rapidly heated to 195°, then slowly, the yellowish anhydrous salt turns a deeper yellow and melts with gas evolution at 196–7°. It is soluble in absolute alcohol, especially on warming, and is almost insoluble in boiling dry acetone or chloroform.

Air dry: Subs., 0.5990: loss, 0.0201 *in vacuo* at room temp. over H_2SO_4.
Calc. for $C_{19}H_{23}ON_2Br.2HBr.H_2O$: H_2O, 3.25. Found: 3.36.
Anhydrous: Subs., 0.1367: 6.6 cc. N (26.0°, 752 mm.).
Subs., 0.1423: 10.71 cc. $AgNO_3$ sol. (1 cc. = 0.00396 g. Br).
Calc. for $C_{19}H_{23}ON_2Br.2HBr$: N, 5.22; Br^-, 29.76. Found: N, 5.45; Br^-, 29.81.

The free base was found to be quite unstable. It is precipitated from aqueous solution by sodium carbonate or bicarbonate as pale yellow, amorphous flocks, but on extracting with chloroform red decomposition products are rapidly formed, and while crystals could be obtained by taking up in dry acetone the residue from the evaporation of the chloroform *in vacuo*, satisfactory analytical values could not be realized. It was also noted that the product contained ionizable halogen. Solution of the dihydrobromide in excess alkali resulted in the rapid development of an orange color, and the base recovered on adding ammonium chloride was found to be halogen-free.

B. *Derivatives of Dihydro-Quinidine.*

Chloro-dihydro-quinidine.—133 g. of dihydro-quinidine dihydro-chloride (anhydrous) were dissolved in about 10 volumes of dry chloroform and converted into chloro-dihydro-quinidine exactly as in the case of the dihydro-quinine derivative. The crude base was dissolved in several volumes of hot ligroin, filtered with the aid of boneblack from a small amount of insoluble material, and seeded while still warm with crystals obtained by dissolving a test portion in ligroin and letting stand. 44.6 g. of the crystalline base were obtained by keeping the solution warm until most of the base had crystallized and then allowing to cool slowly to room temperature. The filtrate was treated with absolute alcohol and neutralized to litmus with alcoholic hydrochloric acid, an additional 21 g. of the hydrochloride separating in this way as delicate, voluminous needles. Recrystallized twice from ligroin as above, the base forms hard, compact, cream-colored aggregates of minute plates which melt at 93.5–5° with preliminary softening and show $[\alpha]_D^{22} + 20.0°$ in absolute alcohol; $c = 0.600$. It gives the thalleoquinine reaction and is very easily soluble in alcohol, acetone, chloroform, benzene, or ether.

Subs., 0.1280: 9.4 cc. N (24.0°, 770 mm.).
Subs., 0.1742: (Carius) AgCl, 0.0717.
Calc. for $C_{20}H_{25}ON_2Cl$: N, 8.13; Cl, 10.28.   Found: N, 8.55; Cl, 10.18.

The Hydrochloride.—The base was dissolved in a little absolute alcohol and neutralized to wet litmus with dry alcoholic hydrochloric acid. The salt which separated was recrystallized from absolute alcohol, forming sheaves and rosets of delicate needles. After drying *in vacuo* $[\alpha]_D^{25}$ was $+ 39.7°$ in water; $c = 0.795$. The salt turns yellow and softens above 205° and blackens and melts at 208–9°. It is quite soluble in cold water and dissolves readily in dry methyl alcohol, less easily in dry alcohol or chloroform, and only with difficulty in boiling dry acetone.

Subs., 0.1191: 6.25 cc. AgNO₃ sol. (1 cc. = 0.00176 g. Cl).
Calc. for $C_{20}H_{25}ON_2Cl.HCl$: Cl⁻, 9.31.   Found: 9.24.

Dihydro-quinidane (*Dihydro-desoxy-quinidine*).—40 g. of chlorodihydro-quinidine were reduced to dihydro-quinidane and isolated

exactly as in the case of the corresponding quinine derivatives. The oily base was dissolved in alcohol, diluted with water to incipient turbidity, and seeded with crystals obtained from a test portion dissolved in a little ligroin and allowed to evaporate spontaneously. The dihydrate separated as glistening rhombs, the amount being increased by the cautious addition of small quantities of water. The yield was 28.7 g. Recrystallized twice from 60% alcohol the dihydrate separates as rhombs and prisms which show $[\alpha]_D^{22.5} = + 167.3°$ in absolute alcohol; $c = 1.124$, and melt at 81–3° with marked preliminary softening to a turbid liquid which clears at 88°. It dissolves readily in alcohol, acetone, chloroform, or benzene, less easily in ether, exhibits a marked purplish fluorescence in dil. sulfuric acid solution, and gives the thalleoquinine test. The anhydrous alkaloid melts at 68.5–70° with preliminary softening.

Air dry: Subs., 0.4798: loss, 0.0496 *in vacuo* at room temp. over H_2SO_4.
Subs., 0.1242: CO_2, 0.3159; H_2O, 0.0980.
Subs., 0.1255: 9.6 cc. N (23.0°, 760 mm.).
Subs., 0.1554: (Kjeldahl) 9.05 cc. 0.1 N HCl.
Cal. for $C_{20}H_{26}ON_2.2H_2O$: C, 69.31; H, 8.73; N, 8.09; H_2O, 10.40. Found: C, 69.37; H, 8.83; N, 8.82, 8.17; H_2O, 10.33.

The Hydrobromide.—The dihydrate was suspended in a little 50% alcohol and neutralized hot with 40% aqueous hydrobromic acid. The salt separated on chilling and rubbing and was recrystallized twice from 50% alcohol, separating as prismatic needles containing one molecule of water of crystallization, dissolving rather sparingly in cold water but readily in cold alcohol, and showing $[\alpha]_D^{24} = + 64.5°$ in water; $c = 1.063$. The anhydrous salt melts slowly at 151–2° with very slight gas evolution and dissolves readily in methyl or ethyl alcohol, acetone, or chloroform and difficultly in cold benzene but easily on warming.

Subs., 0.4948: loss, 0.0222 *in vacuo* at 80° over H_2SO_4.
Calc. for $C_{20}H_{26}ON_2.HBr.H_2O$: H_2O, 4.40. Found: 4.49.
Anhydrous: Subs., 0.1177: 7.5 cc. N (26.0°, 756 mm.).
Subs., 0.1048: 5.45 cc. $AgNO_3$ sol. (1 cc. = 0.00396 g. Br).
Calc. for $C_{20}H_{26}ON_2.HBr$: N, 7.16; Br, 20.42. Found: N, 7.24; Br, 20.59.

The Dihydrobromide.—Dihydro-quinidane dihydrate was dissolved in absolute alcohol and treated with hydrobromic acid (sp. gr. 1.49)

until acid to wet Congo red paper. Dry ether was then added to incipient turbidity and the solution seeded with crystals obtained by precipitating a test portion with dry ether and taking up the product in dry acetone. The collected salt was suspended in a little boiling absolute alcohol containing a few drops of hydrobromic acid and treated on the water bath with small portions of 95% alcohol until clear. The dihydrobromide separated slowly on cooling and seeding as faintly yellow, glistening rhombs which were white when powdered and contained no solvent of crystallization. It begins to turn yellow above 180°, softens at about 210°, and is completely fluid, with slow gas evolution, at about 225°. The salt dissolves readily in water and very difficultly in boiling dry acetone or chloroform.

Subs., 0.1051: 8.91 cc. AgNO$_3$ sol. (1 cc. = 0.00396 g. Br).
Calc. for C$_{20}$H$_{26}$ON$_2$.2HBr: Br, 33.86. Found: 33.59.

Dihydro-quinidane Methiodide.—The salt separates in quantitative yield from a solution of the components in dry acetone. Recrystallized twice from absolute alcohol it forms faintly yellow, warty masses of short needles which dissolve appreciably in cold water, readily on heating. When rapidly heated to 160°, then slowly, it melts at 163–4° with slight preliminary softening to a yellow liquid. It dissolves readily in methyl alcohol or chloroform.

Subs., 0.1020: 4.47 cc. AgNO$_3$ sol. (1 cc. = 0.00629 g. I).
Calc. for C$_{21}$H$_{29}$ON$_2$I: I, 28.07. Found: 27.57.

Dihydro-cupreidane (Dihydro-desoxy-cupreidine).—25 g. of dihydro-quinidane were demethylated as in the case of the quinine analog. The residue from the concentration *in vacuo* crystallized on standing overnight, and was dissolved in much water, the solution made alkaline, and then treated with saturated ammonium chloride solution. The gummy alkaloid was filtered off, dried, and combined with a further amount obtained by extracting the filtrate with ether. A crystalline product was rapidly formed on rubbing with dry acetone, the yield being 16.1 g. after washing with dry acetone. Recrystallized from toluene, in which it is quite soluble at the boiling point, but sparingly so at even a few degrees less, it forms a crust of cream-colored rosets of microcrystals which melt slowly at 183–3.5° to a

straw-colored liquid. $[\alpha]_D^{24}$ is $+$ 183.7° in absolute alcohol; $c = 0.991$. It dissolves readily in methyl or ethyl alcohol or chloroform, and very difficultly in boiling benzene. In its chemical reactions, it behaves like its stereoisomer, dihydrocupreane.

Subs., 0.1033: CO_2, 0.2912; H_2O, 0.0762.
Subs., 0.1428: 11.75 cc. N (23.5°, 764 mm.).
Calc. for $C_{19}H_{24}ON_2$: C, 76.97; H, 8.17; N, 9.46. Found: C, 76.88; H, 8.25; N, 9.52.

The Hydrochloride.—The base was dissolved in a slight excess of N hydrochloric acid, neutralized, and the solution treated with saturated sodium chloride solution. It soon solidified, and the collected salt was recrystallized from water, separating on seeding as short, slightly brownish, glistening needles which contain 2 molecules of water of crystallization when air dry, but appear to separate with more water, as the air dry salt first dissolves freely in water and then quickly crystallizes again. The solution gives a brownish color with ferric chloride. The anhydrous salt gradually softens to a jelly at 125–45°, and forms a yellow fluid at about 200°. It dissolves readily in dry methyl or ethyl alcohol or chloroform, the colorless solutions in the first two turning yellow on adding water. $[\alpha]_D^{22}$ of the anhydrous salt is $+$ 76.8° in water; $c = 0.436$.

Air dry: Subs., 0.5424: loss, 0.0547 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{19}H_{24}ON_2.HCl.2H_2O$: H_2O, 9.77. Found: 10.08.
Anhydrous: Subs., 0.1244: 9.2 cc. N (22.5°, 763 mm.).
Subs., 0.1530: 9.18 cc. $AgNO_3$ sol. (1 cc. $=$ 0.00176 g. Cl).
Calc. for $C_{19}H_{24}ON_2.HCl$: N, 8.42; Cl, 10.66. Found: N, 8.57; Cl, 10.56.

The Dihydrobromide.—The salt separates from a small volume of chilled, dil. hydrobromic acid as faintly yellow aggregates of hexagonal plates containing 2 molecules of water of crystallization and dissolving quite readily in water at room temperature, less easily at 0°. The anhydrous salt softens and turns yellow above 140°, forming a jelly by the time 175° is reached and becoming completely fluid at about 205°. It forms a gum under dry methyl or ethyl alcohol, dissolving on shaking, and also gums under dry chloroform but does not dissolve completely.

Air dry: Subs., 0.5211: loss, 0.0414 *in vacuo* at room temp. over H_2SO_4.
Calc. for $C_{19}H_{24}ON_2.2HBr.2H_2O$: H_2O, 7.29. Found: 7.95.
Anhydrous: Subs., 0.1225: 10.68 cc. AgNO$_3$ sol. (1 cc. = 0.00396 g. Br).
Calc. for $C_{19}H_{24}ON_2.2HBr$: Br, 34.89. Found: 34.54.

Dihydrocupreidane Methiodide.—The salt soon separated from a solution of the components in absolute alcohol. Recrystallized from 25% alcohol it forms short, slightly brownish, prismatic rods which contain no water of crystallization and are difficultly soluble in cold water but readily on heating. It is quite soluble in methyl alcohol or 50% alcohol and almost insoluble in boiling dry acetone or chloroform. $[\alpha]_D^{23}$ is + 95.0° in 50% alcohol; c = 1.105.

Subs., 0.1369: 6.25 cc. AgNO$_3$ sol. (1 cc. = 0.00629 g. I).
Calc. for $C_{20}H_{27}ON_2I$: I, 28.97. Found: 28.71.

Bromo - dihydro - cupreidine Dihydrobromide. — Chloro - dihydro-quinidine hydrochloride was demethylated as in the case of chloro-dihydro-quinine. In less than 2 hours the dihydrobromide separated from the boiling solution and the mixture was accordingly cooled and the salt filtered off, an additional amount being recovered on concentrating the filtrate to small bulk. Recrystallized from water containing a little hydrobromic acid it separates as sheaves of pale yellow, delicate needles which are anhydrous and decompose at about 255–6° with slight preliminary softening and darkening. $[\alpha]_D^{24}$ is − 47.7° in water; c = 1.141. A neutralized aqueous solution gives a brown color with ferric chloride. The salt is sparingly soluble in boiling absolute alcohol, but dissolves somewhat more readily in boiling dry methyl alcohol, and behaves in the same way as its stereoisomer with carbonates and alkalies, the free base being equally unstable.

Subs., 0.1620: 7.40 cc. N (23.0°, 755 mm.).
Subs., 0.1200; 9.0 cc. AgNO$_3$ sol. (1 cc. = 0.00396 g. Br).
Calc. for $C_{19}H_{23}ON_2Br.2HBr$: N, 5.22; Br⁻, 29.76. Found: N, 5.23; Br⁻, 29.70.

If a warm, fairly concentrated solution in water is nearly neutralized with sodium hydroxide, the monohydrobromide separates c rubbing as diamond shaped, microscopic platelets.

C. Derivatives of Quinidine.

Chloroquinidine Hydrochloride.—Chloroquinidine, prepared essentially according to Comstock and Koenigs,[8] was neutralized in dil. alcohol with hydrochloric acid. The solution, after boiling with boneblack and filtering, was concentrated to dryness *in vacuo* and the residue taken up in boiling absolute alcohol. The hydrochloride was deposited on cooling and was recrystallized from absolute alcohol, with the addition of a little dry ether to the cooled solution. The salt forms woolly, cream-colored needles which show $[\alpha]_D^{25} = + 47.7°$ in water; $c = 1.017$. When rapidly heated to 195°, then slowly, it gradually melts and decomposes from 199–206°. It dissolves readily in water with a faint yellow color, and also dissolves easily in dry methyl alcohol, less readily in dry chloroform, and is practically insoluble in dry acetone.

Subs., 0.1278: 8.4 cc. N (29.0°, 752 mm.).
Subs., 0.1216; 6.26 cc. AgNO$_3$ sol. (1 cc. = 0.00181 g. Cl).
Calc. for C$_{20}$H$_{23}$ON$_2$Cl.HCl: N, 7.39; Cl⁻, 9.35.   Found: N, 7.35; Cl⁻, 9.33.

Quinidane (Desoxy-quinidine) Hydrochloride.—Quinidane (desoxy-quinidine[9]) was dissolved in a little absolute alcohol, treated with a molecular equivalent of conc. hydrochloric acid, and then with 6–8 volumes of dry ether. The hydrochloride crystallized on rubbing, and was dissolved in warm 95% alcohol, and the solution treated with 6–8 volumes of dry ether and filtered rapidly from the slight precipitate which carried considerable colored impurity with it. The salt separated slowly on seeding as aggregates of minute crystals which retained solvent equivalent to one molecule of water of crystallization on air drying. The anhydrous salt shows $[\alpha]_D^{30} = + 81.9°$ in water; $c = 1.313°$, and sinters and gradually melts above 125°, forming a yellow liquid at about 145°. It dissolves readily in water, methyl or ethyl alcohol, or chloroform, sparingly in cold, dry acetone, although fairly easily on boiling, and is very difficultly soluble in boiling benzene.

[8] *Ber.*, **18**, 1223 (1885).
[9] Koenigs, *Ber.*, **28**, 3147 (1895); Rabe, *et al.*, *Ann.*, **373**, 107 (1910).

Air dry: Subs., 0.6065: loss, 0.0322 *in vacuo* at 80° over H_2SO_4.
Calc. for $C_{20}H_{24}ON_2.HCl.H_2O$: H_2O, 4.97. Found: 5.31.
Anhydrous: Subs., 0.1575: 11.4 cc. N (25.5°, 759 mm.).
Subs., 0.1196: 6.96 cc. $AgNO_3$ sol. (1 cc. = 0.00181 g. Cl).
Calc. for $C_{20}H_{24}ON_2.HCl$: N, 8.13; Cl, 10.28. Found: N, 8.27; Cl, 10.54.

Quinene Dihydrochloride.—Crude quinene, prepared and isolated according to Comstock and Koenigs,[10] was dissolved in absolute alcohol, treated with an excess of absolute alcoholic hydrochloric acid, and the dihydrochloride precipitated with dry ether. Recrystallized from absolute alcohol it forms sheaves and rosets of delicate, lemon-yellow needles which retain solvent equivalent to one molecule of water of crystallization when air dry. The anhydrous salt shows $[\alpha]_D^{24.5} = +18.4°$ in water; $c = 1.060$, and gradually sinters and darkens when heated, melting to a dark red-brown liquid at 180–5°. It dissolves readily in water or methyl alcohol, less easily in absolute alcohol or dry chloroform, and is practically insoluble in dry acetone.

Air dry: Subs., 0.6382: loss, 0.0267 *in vacuo* at room temp. over H_2SO_4.
Calc. for $C_{20}H_{22}ON_2.2HCl.H_2O$: H_2O, 4.54. Found: 4.18.
Anhydrous: Subs., 0.1329: 8.8 cc. N (25.0°, 756 mm.).
Subs., 0.1297: 13.85 cc. $AgNO_3$ sol. (1 cc. = 0.00176 g. Cl).
Calc. for $C_{20}H_{22}ON_2.2HCl$: N, 7.39; Cl, 18.69. Found: N, 7.55; Cl, 18.81.

SUMMARY.

The so-called "desoxy" derivatives of dihydro-quinine and dihydro-quinidine are described, together with certain of their salts, homologs, and the alkaloidal derivatives encountered in their preparation. It is proposed that the substitution of the suffix "ane" for the prefix "desoxy" would simplify the terminology of this group of substances. The series dihydro-quinine → chlorodihydro-quinine → dihydro-quinane → dihydro-cupreane → ethyl-dihydro-cupreane, resulting in the preparation of an analog of ethyl-dihydro-cupreine (optochin) was prepared and studied.

[10] *Ber.*, 17, 1988 (1884).

RATE OF HYDROLYSIS OF PHOSPHORIC ESTERS OF SUGAR DERIVATIVES.

FIRST PAPER.

BY P. A. LEVENE AND M. YAMAGAWA.

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A number of the components of the tissues contain in their molecules either phosphoric or sulfuric acid. The phosphoric acid radicle is contained in the mono- and polynucleotides, and the sulfuric acid radicle in chondroitin- and mucoitin-sulfuric acids. In all these compounds the acid radicle is linked in ester form to either a carbohydrate or a polyhydric alcohol. On long acquaintance with substances of this character, one comes to realize that the inorganic radicle is linked in different substances of the same group with different degrees of firmness. Thus, for instance, in inosinic- and uridin-phosphoric acids on one hand, and in guanylic on the other; in chondroitin-sulfuric on one hand, and in mucoitin-sulfuric on the other, the organic acids display different degrees of resistance towards hydrolytic agents.

In compounds such as the ribose nucleotides, two components of the molecule, the sugar and the inorganic acid, remain constant, and only the third component is variable. It is evident that the firmness of the union between the acid and the rest of the molecule may be conditioned by two factors: the character of the base, and the position of the acid radicle on the sugar molecule.

There exists no systematic study of the rate of hydrolysis of ester from derivatives of sugars which have only one point of difference; namely, that of the allocation of the acid radicle. However, in the preparative work on sugar derivatives by Fischer[1] and his students, and by Irvine[2] and his students, one finds abundant evidence that such differences in the rate of hydrolysis exist.

[1] Fischer, E., and Noth, H., *Ber. chem. Ges.*, 1918, li, 321.

[2] Irvine, J. C., and Scott, J. P., *J. Chem. Soc.*, 1913, ciii, 575.

A more detailed as well as a more systematic investigation of this problem is much desired not only for purely academic considerations, but also for what, in a way, may be regarded as practical or applied reasons.

It is a difficult task to determine the allocation of an acyl group on the sugar molecule by direct chemical method. On few occasions the method was successful, and only then when the substitution took place on the primary alcoholic group. When any of the secondary alcoholic groups are substituted the allocation of the substituting group has thus far not been successful. As yet the present work does not embrace a sufficiently large number of esters, and is offered in its present form because one of the authors is compelled to discontinue his cooperation.

The substances[3] employed in this work were phosphoric esters of: (1) α-methylglucoside; (2) β-, γ-, ε-trimethyl methylglucoside; (3) α-, β-, γ-, ε-diacetone glucose; (4) α-, β-monoacetone glucose; (5) a phosphoric ester of the same sugar derivative as in (4), but differing from the substance (4) by the position of the phosphoric acid radicle; (6) ʃ-benzoyl-α-β-monoacetone glucose.

In the first, the position of the phosphoric acid radicle is not established, and the interest attached to the substance is due primarily to the fact that the sugar radicle in it is non-substituted. In the following two substances, namely β-, γ-, ε-trimethyl methylglucoside and diacetone glucose, the phosphoric acid radicle is attached to the same carbon atom, and hence the two substances differ in the nature and in the molecular weight of the substituting groups. Differences in the rate of hydrolysis of these two substances should be attributed to the influence of these groups.

The fourth substance differed from the fifth in the method of preparation. Whereas the latter was obtained by the action of phosphorous oxychloride on monoacetone glucose, the former is formed as a by-product by the action of phosphorous oxychloride on diacetone glucose. Apparently in the former substance the phosphoric acid radicle was linked to the primary alcoholic group,

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[3] The preparation of these substances will be described in a subsequent paper by P. A. Levene and G. M. Meyer.

whereas in the latter it was linked to one of the secondary alcoholic groups.

The sixth substance, ζ-benzoyl-α-β-monoacetone glucosidophosphoric acid, differs from the preceding two by the position of the phosphoric acid radicle, by the number of the substituting groups, and by the difference in character of one of the substituting groups.

The hydrolysis of these substances was carried out in approximately equivalent concentration, which was in the neighborhood of 10 per cent. As catalytic agent 0.1 N sulfuric acid was employed. The temperature of hydrolysis was 100°C.

As was anticipated, the rate of hydrolysis followed the monomolecular law. The constants of hydrolysis $K = \dfrac{1}{t} \log \dfrac{a}{a-x}$ were as follows:

$$K_1 = 22 \ (10^{-2})$$
$$K_2 = 43 \ (10^{-2})$$
$$K_3 = 56 \ (10^{-2})$$
$$K_4 = 44 \ (10^{-2})$$
$$K_5 = 58 \ (10^{-2})$$
$$K_6 = 18 \ (10^{-2})$$

(The subscripts indicate the substances in the order of their tabulation.)

Thus in this series of substances apparently both the position of the phosphoric acid radicle and the nature of the substituting groups exert an influence on the stability of the phosphoric ester linking.

On the other hand, the constants of hydrolysis of the sugar derivatives which differ from one another only in point of position of the acid radicle, as Substances 4 and 5, are determined by the position of the inorganic radicle.

It was expected that the position of the phosphoric acid radicle would play the determining part in the rate of hydrolysis. This expectation has not materialized, and it will therefore be necessary to prepare such phosphoric esters of glucose or of methylglucoside which will have the inorganic radicle in a known position, and which will be free from other substituting groups.

It is planned to continue the work in this direction in this laboratory.

α-Methylglucosidophosphoric Acid.—11.644 gm. of barium methyl-glucosidophosphate were ground up with a little water, 49.2 cc. of N H_2SO_4 were added, and the volume was made up to 100 cc. The solution was filtered from the barium sulfate without further addition of water.

The concentration of methylglucosidophosphoric acid was established by a polariscopic determination; 5 cc. of the solution contained 0.450 gm. of methylglucosidophosphoric acid, representing 0.0509 gm. of P.

5 cc. of this solution were pipetted into glass tubes together with 5 cc. of 0.2 N H_2SO_4. The tubes were sealed and heated in an oil bath at 100°C. for various intervals as indicated in the tables. After cooling, the contents of each tube were made up to 100 cc. and the phosphorus was determined as magnesium pyrophosphate on 40 cc. of this solution.

The results are tabulated in Tables I, Ia, II, and IIa.

β-γ-ε-Trimethyl Methylglucosido-ζ-Phosphoric Acid.—13.0785 gm. of the barium salt were ground up in a little water, the barium was precipitated by 42.7 cc. of N H_2SO_4, and the solution made up to 100 cc. 5 cc. of this solution contained 0.45 gm. of trimethyl methyl-glucosidophosphoric acid, determined polarimetrically, equivalent to 0.0441 gm. of phosphorus.

5 cc. of the filtrate were heated in sealed tubes with 5 cc. of 0.2 N H_2SO_4 for various periods at 100° and the phosphorus was determined as magnesium pyrophosphate.

The results are tabulated in Tables III, IIIa, IV, and IVa.

α-β-γ-ε-Diacetone Glucosido-ζ-Phosphoric Acid.—10.1805 gm. of the barium salt were dissolved in a small volume of water and made up to 75 cc. 5 cc. of this solution, containing 0.450 gm. of diacetone glucoside phosphoric acid, equivalent to 0.041 gm. of phosphorus, were pipetted into glass tubes together with 3.3 cc. of N H_2SO_4 and 1.7 cc. of water. The tubes were sealed and heated at 100° for various intervals.

The results are tabulated in Tables V and Va.

α-β-Monoacetone Glucosido-ζ-Phosphoric Acid (from Diacetone Glucose).—7.5617 gm. of the barium salt of this substance were dissolved in a small volume of warm water and made up to 50 cc. Of this solution 3 cc., equivalent to 0.029 gm. of P, were pipetted into glass tubes together with 2.27 cc. of N H_2SO_4 and 0.73 cc. of water and sealed. The tubes were heated at 100° for various intervals.

The results are tabulated in Tables VI and VIa.

α-β-Monoacetone Glucosidophosphoric Acid (from Monoacetone Glucose).—5.876 gm. of the barium salt of the substance were dissolved in a small quantity of warm water and made up to 40 cc. 3 cc. of this solution, equivalent to 0.028 gm. of P, together with 2.29 cc. of N H_2SO_4 and 0.71 cc. of water were heated in sealed tubes for various intervals.

The results are tabulated in Tables VII and VIIa.

ζ-Benzoyl-α-β-Monoacetone Glucosidophosphoric Acid.—8.4285 gm. of the barium salt of the substance were rubbed up with a small volume of warm water, made up to 75 cc., and filtered. 5 cc. of the filtrate, equivalent to 0.035 gm. of P, together with 2.97 cc. of N H_2SO_4 and 2.03 cc. of water were heated in sealed tubes for various intervals.

The results are tabulated in Tables VIII and VIIIa.

TABLE I.

α-Methylglucosidophosphoric Acid.

Time.	Mg₂P₂O₇	Average.	P in Mg₂P₂O₇.	P in free acid.	P in total P.
hrs.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
1	0.0015 0.0016	0.0016	0.0004	0.24	2.16
	0.0017 0.0016	0.0017	0.0005	0.27	2.36
2	0.0043 0.0045	0.0044	0.0012	0.69	6.09
	0.0044 0.0045	0.0045	0.0013	0.69	6.09
4	0.0081 0.0083	0.0082	0.0023	1.27	11.20
	0.0085 0.0088	0.0087	0.0024	1.34	11.98
8	0.0175 0.0171	0.0173	0.0048	2.69	23.77
	0.0177 0.0178	0.0178	0.0050	2.76	24.36
16	0.0296 0.0292	0.0294	0.0082	4.56	40.27
	0.0304 0.0294	0.0299	0.0083	4.62	40.86
24	0.0394 0.0396	0.0395	0.0110	6.11	54.03
	0.0396 0.0398	0.0397	0.0111	6.11	54.03

TABLE II.

α-Methylglucosidophosphoric Acid.

Time.	$Mg_2P_2O_7$	Average.	P in $Mg_2P_2O_7$.	P in free acid.	P in total P.
hrs.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
1	0.0014 0.0016	0.0015	0.0004	0.24	2.16
	0.0018 0.0016	0.0017	0.0005	0.27	2.36
2	0.0044 0.0042	0.0043	0.0012	0.67	5.89
	0.0042 0.0046	0.0044	0.0012	0.69	6.09
4	0.0082 0.0081	0.0082	0.0023	1.27	11.20
	0.0087 0.0086	0.0087	0.0024	1.33	11.79
8	0.0170 0.0170	0.0170	0.0047	2.62	23.18
	0.0170 0.0170	0.0170	0.0047	2.62	23.18
16	0.0304 0.0308	0.0306	0.0085	4.78	42.24
	0.0314 0.0315	0.0315	0.0088	4.89	43.22
24	0.0409 0.0406	0.0408	0.0114	6.31	55.78
	0.0417 0.0418	0.0418	0.0116	6.47	57.17
32	0.0504 0.0504	0.0504	0.0140	7.80	68.96
	0.0500 0.0502	0.0501	0.0140	7.76	68.56

TABLE III.

β-γ-ε-Trimethyl Methylglucosido-ζ-Phosphoric Acid.

Time.	Mg₂P₂O₇	Average.	P in Mg₂P₂O₇.	P in free acid.	P in total P.
hrs.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
1	0.0025 0.0025	0.0025	0.0007	-0.38	3.83
	0.0026 0.0026	0.0026	0.0007	0.40	4.05
2	0.0071 0.0069	0.0070	0.0020	1.09	11.04
	0.0060 0.0068	0.0068	0.0019	1.04	10.59
4	0.0124 0.0125	0.0125	0.0035	1.93	19.60
	0.0129 0.0120	0.0125	0.0035	1.93	19.60
8	0.0247 0.0247	0.0247	0.0069	3.82	38.74
	0.0242 0.0240	0.0241	0.0067	3.73	37.84
16	0.0403 0.0402	0.0403	0.0112	6.22	63.06
	0.0410 0.0412	0.0411	0.0115	6.36	64.42
24	0.0527 0.0526	0.0527	0.0147	8.16	82.66
	0.0524 0.0527	0.0526	0.0147	8.13	82.43

TABLE IV.

β-γ-ε-Trimethyl Methylglucosido-ς-Phosphoric Acid.

Time.	$Mg_2P_2O_7$	Average.	P in $Mg_2P_2O_7$.	P in free acid.	P in total P.
hrs.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
1	0.0028 0.0026	0.0027	0.0008	0.42	4.24
	0.0026 0.0025	0.0026	0.0007	0.40	4.08
2	0.0071 0.0074	0.0073	0.0020	1.13	11.46
	0.0068 0.0070	0.0069	0.0019	1.07	10.83
4	0.0130 0.0130	0.0130	0.0036	2.01	20.40
	0.0125 0.0130	0.0128	0.0036	1.98	20.09
8	0.0245 0.0246	0.0246	0.0069	3.81	38.60
	0.0246 0.0245	0.0245	0.0068	3.79	38.45
16	0.0388 0.0390	0.0389	0.0108	6.02	61.04
	0.0386 0.0390	0.0388	0.0108	6.00	60.81
24	0.0496 0.0492	0.0494	0.0138	7.64	77.48
	0.0492 0.0494	0.0493	0.0137	7.62	77.25

TABLE V.

α-β-γ-ε-Diacetone Glucosido-ζ-Phosphoric Acid.

Time.	$Mg_2P_2O_7$	Average.	P in $Mg_2P_2O_7$.	P in free acid.	P in total P.
hrs.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
1	0.0040 0.0040	0.0040	0.0011	0.62	6.80
	0.0037 0.0040	0.0039	0.0011	0.62	6.80
2	0.0090 0.0085	0.0088	0.0025	1.36	14.95
	0.0091 0.0090	0.0091	0.0025	1.41	15.46
4	0.0159 0.0157	0.0157	0.0044	2.43	26.68
	0.0154 0.0158	0.0156	0.0043	2.42	26.51
8	0.0262 0.0262	0.0262	0.0073	4.06	44.52
	0.0262 0.0262	0.0262	0.0073	4.06	44.52
16	0.0419 0.0438	0.0429	0.0117	6.64	72.90
	0.0419 0.0394	0.0419	0.0117	6.49	71.20
24	0.0525 0.0525	0.0525	0.0146	8.13	89.22
	0.0525 0.0525	0.0525	0.0146	8.13	89.22

TABLE VI.

α-β-Monoacetone Glucosido-ζ-Phosphoric Acid (from Diacetone Glucose).

Time.	$Mg_2P_2O_7$	Average.	P in $Mg_2P_2O_7$.	P in free acid.	P in total P.
hrs.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
1	0.0050 0.0047	0.0049	0.0014	0.78	7.56
	0.0048 0.0047	0.0048	0.0014	0.78	7.56
2	0.0103 0.0097	0.0100	0.0028	1.56	15.12
	0.0100 0.0100	0.0100	0.0028	1.56	15.12
4	0.0183 0.0180	0.0182	0.0051	2.84	27.65
	0.0175 0.0173	0.0174	0.0048	2.67	25.92
8	0.0323 0.0323	0.0323	0.0090	5.00	48.60
	0.0320 0.0320	0.0320	0.0089	4.96	48.16
16	0.0483 0.0483	0.0483	0.0135	7.51	73.00
	. . . . . . 0.0483	0.0483	0.0135	7.51	73.00
24	0.0570 0.0570	0.0570	0.0159	8.84	85.96
	0.0570 0.0570	0.0570	0.0159	8.84	85.96

TABLE VII.

α-β-Monoacetone Glucosidophosphoric Acid (from Monoacetone Glucose).

Time.	Mg₂P₂O₇	Average.	P in Mg₂P₂O₇.	P in free acid.	P in total P.
hrs.	gm.	gm.	gm.	per cent	per cent
1	0.0033 0.0038	0.0036	0.0010	0.55	5.40
	0.0035 0.0037	0.0036	0.0010	0.55	5.40
2	0.0073 0.0080	0.0077	0.0021	1.18	11.45
	0.0075 0.0077	0.0076	0.0021	1.18	11.45
4	0.0150 0.0153	0.0152	0.0042	2.33	22.68
	0.0143 0.0145	0.0144	0.0040	2.22	21.60
8	0.0260 0.0270	0.0265	0.0074	4.11	39.96
	0.0268 0.0261	0.0265	0.0074	4.11	39.96
16	0.0405 0.0408	0.0407	0.0113	6.29	61.12
	0.0403 0.0403	0.0403	0.0112	6.22	60.47
24	0.0511 0.0511	0.0511	0.0142	7.89	76.67
	0.0508 0.0510	0.0509	0.0142	7.89	76.67

TABLE VIII.

ζ-Benzoyl-α-β-Monoacetone Glucosidophosphoric Acid.

Time.	Mg₂P₂O₇	Average.	P in Mg₂P₂O₇.	P in free acid.	P in total P.
hrs.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
1	0.0012 0.0012	0.0012	0.0003	0.19	2.42
	0.0012 0 0012	0 0012	0.0003	0.19	2.42
2	0.0022 0.0022	0.0022	0.0006	3.40	4.42
	0.0021 0.0021	0.0021	0.0006	3.24	4.42
4	0.0048 0.0048	0.0048	0.0013	7.42	9.65
	0.0048 0.0048	0.0048	0.0013	7.42	9.65
8	0.0094 0.0094	0.0094	0.0026	14.56	18.93
	0.0094 0.0094	0.0094	0.0026	14.56	18.93
16	0.0174 0.0173	0.0174	0.0048	26.93	35.03
	0.0174 0.0174	0.0174	0.0048	26.93	35.03
24	0.0223 0.0223	0.0223	0.0062	34.53	44.92
	0.0223 0.0223	0.0223	0.0062	34.53	44.92

Velocity Constants.

TABLE I*a*.

α-Methylglucosidophosphoric Acid.

	Mg$_2$P$_2$O$_7$ (x)	$a - x$	$\frac{1}{t} \log \frac{a}{a-x}$
min.	*gm.*		
0			
60	0.0017	0.0714	0.00017
120	0.0045	0.0686	0.00023
240	0.0085	0 0646	0.00022
480	0.0176	0.0555	0.00025
960	0.0297	0.0434	0.00023
1,440	0.0396	0.0335	0.00023
	$a = 0.0731$		
Average..			0.00022

TABLE II*a*.

α-Methylglucosidophosphoric Acid.

t	Mg$_2$P$_2$O$_7$ (x)	$a-x$	$\frac{1}{t} \log \frac{a}{a-x}$
min.	*gm.*		
0			
60	0.0016	0.0715	0.00016
120	0.0044	0.0687	0.00022
240	0.0085	0.0646	0.00022
480	0.0170	0.0561	0.00023
960	0.0311	0.0420	0.00025
1,440	0.0413	0.0318	0.00025
1,920	0.0503	0.0228	0.00026
	$a = 0.0731$		
Average..			0.00022

TABLE IIIa.

β-γ-ε-*Trimethyl Methylglucosido-ζ-Phosphoric Acid.*

t	$Mg_2P_2O_7$ (x)	$a-x$	$\frac{1}{t}\log\frac{a}{a-x}$
min.	*gm.*		
0			
60	0.0026	0.0608	0.00030
120	0.0069	0.0565	0.00042
240	0.0125	0.0509	0.00040
480	0.0244	0.0390	0.00044
960	0.0407	0.0227	0.00046
1,440	0.0527	0.0107	0.00053
	$a = 0.0634$		
Average..			0.00044

TABLE IVa.

β-γ-ε-*Trimethyl Methylglucosido-ζ-Phosphoric Acid.*

t	$Mg_2P_2O_7$ (x)	$a-x$	$\frac{1}{t}\log\frac{a}{a-x}$
min.	*gm.*		
0			
60	0.0027	0.0607	0.00032
120	0.0071	0.0563	0.00043
240	0.0129	0.0505	0.00041
480	0.0246	0.0388	0.00044
960	0.0389	0.0245	0.00043
1,440	0.0494	0.0140	0.00045
	$a = 0.0634$		
Average..			0.00041

TABLE Va.

α-β-γ-ϵ-*Diacetone Glucosido-ζ-Phosphoric Acid.*

t	$Mg_2P_2O_7$ (x)	$a-x$	$\frac{1}{t}\log\frac{a}{a-x}$
min.	*gm.*		
0			
60	0.0040	0.0549	0.00050
120	0.0090	0.0499	0.00060
240	0.0157	0.0432	0.00056
480	0.0262	0.0327	0.00053
960	0.0424	0.0165	0.00058
1,440	0.0525	0.0064	0.00067
	$a = 0.0589$		
Average...			0.00056

TABLE VIa.

α-β-*Monoacetone Glucosido-ζ-Phosphoric Acid (from Diacetone Glucose).*

t	$Mg_2P_2O_7$ (x)	$a-x$	$\frac{1}{t}\log\frac{a}{a-x}$
min.	*gm.*		
0			
, 60	0.0049	0.06168	0.000553
120	0.0100	0.05658	0.000588
240	0.0178	0.04878	0.000563
480	0.0322	0.03438	0.000598
960	0.0483	0.01828	0.000585
1,440	0.0570	0.00958	0.000585
	$a = 0.06658$		
Average....................................			0.000579

TABLE VIIa.

α-β-Monoacetone Glucosidophosphoric Acid (from Monoacetone Glucose).

t	$Mg_2P_2O_7$ (x)	$a-x$	$\frac{1}{t}\log\frac{a}{a-x}$
min.	*gm.*		
0			
60	0.0036	0.06298	0.000400
120	0.0077	0.05888	0.000445
240	0.0148	0.05178	0.000455
480	0.0265	0.04008	0.000459
960	0.0405	0.02608	0.000423
1,440	0.0510	0.01558	0.000438
	$a = 0.06658$		
Average...			0.000437

TABLE VIIIa.

ζ-Benzoyl-α-β-Monoacetone Glucosidophosphoric Acid.

t	$Mg_2P_2O_7$ (x)	$a-x$	$\frac{1}{t}\log\frac{a}{a-x}$
min.	*gm.*		
0			
60	0.0012	0.0485	0.000177
120	0.0022	0.0475	0.000164
240	0.0048	0.0449	0.000183
480	0.0094	0.0403	0.000189
960	0.0174	0.0323	0.000194
1,440	0.0223	0.0274	0.000180
	$a = 0.0497$		
Average...			0.000181

HYDROLYSIS OF NUCLEOTIDES.

BY M. YAMAGAWA.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

Early in the work on nucleotides it was observed that individual members of this group differed in their resistance to the action of hydrolytic agents. Owing to this peculiarity, Levene and Mandel obtained on hydrolysis of thymus nucleic acid a mononucleotide: thymidinphosphoric acid.[1] Later, Levene, and Levene and Jacobs[2] prepared the pyrimidin nucleotides of yeast nucleic acid and Levene and Jacobs,[3] on hydrolysis of thymus nucleic acid, prepared hexocytidin- and hexothymidin-diphosphoric acids. Jones[4] and his coworkers furnished further observations demonstrating this peculiarity of different nucleotides.

The structural details of the molecule which are responsible for this difference in the behavior of the individual mononucleotides are as yet unknown.

Referring only to the ribose nucleotides, the observations are as follows: Inosinic, uridinphosphoric, and cytidinphosphoric acids demonstrated a higher resistance, whereas adenosinphosphoric and guanosinphosphoric acids possess a lower resistance towards hydrolytic agents.

It is certain that all these nucleotides differ from one another in the nature of the base, but it is also possible that they differ in the position of the phosphoric acid radicle on the carbohydrate.

In only one of these nucleotides, in inosinic acid, is the position of the phosphoric acid known; namely, it is linked to the fifth carbon atom. Whether the stability of this substance is conditioned by the

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[1] Levene, P. A., and Mandel, J. A., *Ber. chem. Ges.*, 1908, xli, 1905.

[2] Levene, P. A., *Biochem. Z.*, 1909, xvii, 120. Levene, P. A., and Jacobs, W., *Ber. chem. Ges.*, 1911, xliv, 1027.

[3] Levene, P. A., and Jacobs, W., *J. Biol. Chem.*, 1912, xii, 411.

[4] Jones, W., and Read, B. E., *J. Biol. Chem.*, 1917, xxix, 123.

position of the phosphoric acid radicle cannot as yet be stated.   There are indications that this may be so, since the ribose phosphoric acid obtained from this nucleotide also possesses considerable stability.

The differences in the stability hitherto observed are only of a qualitative nature.   However, in order that this property of nucleotides may be made the basis for suggestions regarding their structure, it is necessary to obtain more detailed information concerning the comparative rate of hydrolysis of the various nucleotides.

The substances employed in this investigation were as follows: Yeast nucleic acid (employed in form of its barium salt), inosinic acid (in form of barium salt), uridinphosphoric acid (barium salt), guanylic acid (crystalline nucleotide), adenylic acid (crystalline nucleotide), and hexothymidindiphosphoric acid (barium salt).

These substances were employed in approximately equivalent concentrations, and the hydrolysis was accomplished by 0.1 N sulfuric acid at a temperature of 100°C.   It was found by Levene and the writer that consistent results were obtained only when the hydrolysis was carried out in sealed tubes.   The rate of hydrolysis followed, as was expected, the monomolecular law and the constant of hydrolysis $K = \dfrac{1}{t} \log \dfrac{a}{a - x}$ was as follows:

	$\frac{1}{t} \log \frac{a}{a-x}$
Nucleic acid	130 (10^{-2})
Guanosinphosphoric acid	177 (10^{-2})
Adenosinphosphoric "	166 (10^{-2})
Uridinphosphoric "	480 (10^{-3})
Inosinic acid	470 (10^{-3})
Hexothymidindiphosphoric acid	726 (10^{-3})

Thus the velocity constants of the guanosinphosphoric and adenosinphosphoric acids are identical.   On the other hand the constants of inosinic- and uridin-phosphoric are also identical in spite of the fact that their basic component is of a different nature.

EXPERIMENTAL.

Nucleic Acid.—4.1184 gm. of barium nucleate were ground up in a small quantity of water and 16.3 cc. of N H_2SO_4, made up to 30 cc., and filtered. 5 cc. of this solution by Kjeldahl determination contained 0.4184 gm. of nucleic acid equivalent to 0.0397 gm. of phosphorus.

5 cc. of this solution were hydrolyzed with 5 cc. of 0.2 N H_2SO_4 in sealed tubes at 100° for various intervals. The phosphorus was estimated as magnesium pyrophosphate. The results are tabulated in Tables I and I*a*.

In Series 2 and 3, 0.5607 gm. of barium nucleate was weighed off for each tube, equivalent to 0.450 gm. of nucleic acid, and this was hydrolyzed with 1.61 cc. of N H_2SO_4, and 3.39 cc. of water. The contents of these tubes after heating were treated as previously described. The results are tabulated in Tables II, II*a*, III, and III*a*.

Guanosinphosphoric Acid.—6.75 gm. of guanylic acid were dissolved in a small quantity of warm water and made up to 75 cc. By analysis of a sample of this material each 5 cc. of the above solution contained 0.4321 gm. of guanylic acid or an equivalent of 0.0365 gm. of phosphorus. 5 cc. of the guanylic acid solution were hydrolyzed with 5 cc. of 0.2 N H_2SO_4 at 100° for various intervals.

The results are tabulated in Tables IV and IV*a*.

Adenosinphosphoric Acid.—4.733 gm. of adenylic acid are dissolved in a small quantity of warm water and made up to 50 cc.

3 cc. of this solution equivalent to 0.024 gm. of P and 3 cc. of 0.2 N H_2SO_4 are hydrolyzed in sealed tubes at 100° for various intervals.

The results are tabulated in Tables V and V*a*.

Uridinphosphoric Acid.—*Series I.*—10.5404 gm. of barium uridinphosphate were taken up in water and 44.3 cc. of N H_2SO_4. The solution was filtered and 5 cc. of the filtrate containing 0.450 gm. of uridinphosphoric acid (Kjeldahl) equivalent to 0.044 gm. of P are hydrolyzed with 5 cc. of 0.2 N H_2SO_4 at 100°C. for various intervals. The results are tabulated in Tables VI and VI*a*.

Series II.—9.6435 gm. of barium uridinphosphate are taken up in a little water and 41.6 cc. of N H_2SO_4 and further treated as in the previous series. Results are tabulated in Tables VII and VII*a*.

Inosinphosphoric Acid.—11.9936 gm. of barium inosinate were dissolved in a small quantity of water and the barium was precipitated with 38.2 cc. of N H_2SO_4. The solution was made up to 75 cc. and filtered. An analysis of the solution (Kjeldahl) showed 5 cc. of solution to contain 0.4453 gm. of inosinic acid equivalent to 0.0397 gm. of phosphorus.

5 cc. of the filtrate were hydrolyzed with 5 cc. of 0.2 N H_2SO_4 at 100°C. for various periods. The results are tabulated in Tables VIII and VIIIa.

Hexothymidindiphosphoric Acid.—9.893 gm. of the barium salt were ground up in a little water and 24.4 cc. of 2 N H_2SO_4 and the volume was made up to 50 cc. The solution was filtered and of the filtrate 3 cc. were hydrolyzed with 3 cc. of 0.2 N H_2SO_4 at 100°C. for various intervals. The concentration of the material was established by means of a Kjeldahl nitrogen determination.

3 cc. of the filtrate were equivalent to 0.03947 gm. of phosphorus. The results are tabulated in Tables IX and IXa.

TABLE I.

Nucleic Acid.

Time.	$Mg_2P_2O_7$	Average.	P in $Mg_2P_2O_7$.	P in free acid.	P in total P.
hrs.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
1	0.0089 0.0088	0.0089	0.0025	1.48	15.62
	0.0090 0.0088	0.0089	0.0025	1.48	15.62
4	0.0308 0.0306	0.0307	0.0086	5.11	53.90
	0.0309 0.0310	0.0310	0.0086	5.16	54.41

TABLE II.

Nucleic Acid.

Time.	Mg$_2$P$_2$O$_7$	Average.	P in Mg$_2$P$_2$O$_7$.	P in free acid.	P in total P.
hrs.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
1	0.0062 0.0062	0.0062	0.0017	0.94	14.19
	0.0062 0.0077	0.0062	0.0017	0.94	14.19
2	0.0135 0.0135	0.0135	0.0038	2.11	31.73
	0.0149 0.0144	0.0144	0.0040	2.22	33.40
4	0.0251 0.0248	0.0250	0.0070	3.89	58.44
	0.0253 0.0254	0.0254	0.0071	3.94	59.28
8	0.0363 0.0357	0.0360	0.0100	5.56	83.49
	0.0368 0.0370	0.0369	0.0103	5.72	85.99
16	0.0437 0.0434	0.0436	0.0122	6.78	101.86
	0.0440 0.0440	0.0440	0.0123	6.78	101.86
24	0.0458 0.0459	0.0459	0.0128	7.11	106.87
	0.0467 0.0462	0.0465	0.0130	7.22	108.54

TABLE III.

Nucleic Acid.

Time.	Mg$_2$P$_2$O$_7$	Average.	P in Mg$_2$P$_2$O$_7$.	P in free acid.	P in total P.
hrs.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
1	0.0057 0.0059	0 0058	0.0016	0.89	13.36
	0.0059 0.0061	0.0060	0.0017	0.94	14.19
2	0.0133 0.0136	0.0135	0 0038	2.11	31.73
	0.0132 0.0130	0 0131	0.0037	2.06	30.89
4	...... 0.0252	0.0252	0.0070	3.89	58.44
	0.0254 0.0256	0.0255	0 0071	3.94	59.28
'8	0.0364 0.0364	0.0364	0.0101	5.56	83.49
	0.0366 0.0372	0.0369	0.0103	5.72	85.99
16	0.0438 0.0438	0.0438	0.0122	6.78	101.86
	0.0436 0.0434	0.0435	0.0121	6.72	101.02

TABLE IV.

Guanosinphosphoric Acid.

Time	Mg₂P₂O₇	Average.	P in Mg₂P₂O₇	P in free acid.	P in total P.
hrs.	gm.	gm.	gm.	per cent	per cent
1	0.0098 0.0098	0 0098	0.0027	1.57	18.43
	0.0096 0 0096	0.0096	0.0027	1.55	18.16
2	0 0200 0.0201	0.0200	0.0056	3.22	37.67
	0 0198	0 0198	0.0055	3.19	37.40
4	0.0338 0 0344	0 0341	0.0095	5.51	64.50
	0 0337 0 0336	0.0337	0.0094	5.44	63.67
8	0.0449 0.0451	0.0450	0.0125	7.27	85.09
	0 0447 0.0449	0 0448	0 0125	7.22	84.55
12	0.0490 0.0485	0.0488	0.0136	7.87	92.14
	0.0490 0.0485	0.0488	0.0136	7.87	92.14
16	0.0495 0.0497	0.0496	0.0138	8.01	93.77
	0.0493 0.0493	0.0493	0.0137	7.94	92.95
24	0.0507 0.0507	0.0507	0.0141	8.19	95.94
	0.0504 0.0505	0.0505	0.0141	8.15	95.39

TABLE V.

Adenosinphosphoric Acid.

Time.	$Mg_2P_2O_7$	Average.	P in $Mg_2P_2O_7$.	P in free acid.	P in total P
hrs.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent.*
1	0.0110 0.0110	0.0110	0.0031	1.71	19.15
	0.0110 0.0110	0.0110	0.0031	1.71	19.15
2	0.0223 0.0223	0.0223	0.0062	3.44	38.56
	0.0218 0.0230	0.0224	0.0062	3.44	38.56
4	0.0372 0.0370	0.0371	0.0103	5.73	. 64.18
	0.0375 0.0375	0.0375	0.0105	5.84	65.42
8	0.0475 0.0478	0.0477	0.0133	7.40	82.84
	0.0482 0.0475	0.0479	0.0133	7.40	82.84
16	0.0537 0.0537	0.0537	0.0150	8.33	93.28
	0.0535 0.0537	0.0536	0.0149	8.29	92.79
24	0.0540 0.0547	0.0544	0.0152	8.44	94.53
	0.0538 0.0548	0.0543	0.0152	8.44	94.53

TABLE VI.
Uridinphosphoric Acid.

Time.	Mg$_2$P$_2$O$_7$	Average.	P in Mg$_2$P$_2$O$_7$.	P in free acid.	P in total P.
hrs.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
1	0.0030 0.0030	0.0030	0.0008	0.45	4.75
	0.0029 0.0030	0.0030	0.0008	0.45	4.75
2	0.0074 0.0074	0.0074	0.0021	1.13	11.76
	...... 0.0074	0.0074	0.0021	1.13	11.76
4	0.0120 0.0136	0.0136	0.0038	2.06	21.49
	0.0134 0.0137	0.0136	0.0038	2.06	21.49
8	0.0257 0.0253	0.0255	0.0071	3.85	40.27
	0.0257 0.0263	0.0260	0.0072	3.92	40.95
16	0.0415 0.0415	0.0415	0.0116	6.26	65.38
	0.0417 0.0421	0.0419	0.0117	6.32	66.06
24	0.0512 0.0512	0.0512	0.0143	7.73	80.77
	0.0513 0.0513	0.0513	0.0143	7.73	80.77

TABLE VII.

Uridinphosphoric Acid.

Time.	Mg$_2$P$_2$O$_7$	Average.	P in Mg$_2$P$_2$O$_7$.	P in free acid.	P in total P.
hrs.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
1	0.0034 0.0034	0.0034	0.0009	0.57	5.91
	0.0036 0.0036	0.0036	0.0010	0.59	6.16
2	0.0070	0.0070	0.0020	1.16	12.07
	0.0076 0.0074	0.0075	0.0021	1.23	12.81
4	0.0136	0.0136	0.0038	2.24	23.40
	0.0138 0.0137	0.0138	0.0038	2.26	23.64
8	0.0236	0.0236	0.0066	3.87	40.39
	0.0236 0.0238	0.0237	0.0066	3.89	40.64
16	...... 0.0407	0.0407	0.0113	6.70	69.95
	0.0413 0.0410	0.0412	0.0115	6.77	70.69

TABLE VIII.

Inosinphosphoric Acid.

Time.	Mg₂P₂O₇	Average.	P in Mg₂P₂O₇.	P in free acid.	P in total P.
hrs.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
1	0.0033 0.0033	0.0033	0.0009	0.52	5.79
	0.0035 0.0035	0.0035	0.0010	0.55	6.05
2	0.0059 0.0060	0.0060	0.0017	0.94	10.53
	0.0062 0 0062	0.0062	0.0017	0.97	10.88
4	0.0135 0.0132	0.0134	0.0037	2.08	23.42
	0.0139 0.0137	0.0138	0.0038	2.16	24.18
8	0.0219 0.0220	0.0220	0.0061	3.44	38.54
	0.0215 0.0210	0.0213	0.0059	3.32	37.28
16	0.0362 0.0362	0.0362	0.0101	5.66	63.48
	0.0365 0.0363	0.0364	0.0101	5.70	63.98
24	0.0463 0.0457	0.0460	0.0128	7.21	80.86
	0.0457 0.0459	0.0458	0.0128	7.16	80.35

TABLE IX.

Hexothymidindiphosphoric Acid.

Time.	Mg$_2$P$_2$O$_7$	Average.	P in Mg$_2$P$_2$O$_7$.	P in free acid.	P in total P.
hrs.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
1	0.0054 0.0052	0.0053	0.0015	1.37	9.37
	0.0051 0.0055	0.0053	0 0015	1.37	9.37
2	. . . . . . 0.0104	0.0104	0.0029	2.67	18.23
	0.0103 0.0104	0.0104	0.0029	2.67	18.23
4	0.0189 0.0181	0.0185	0.0052	4.78	32.66
	0.0183 0.0186	0.0185	0 0052	4.78	32.66
8	0.0314 0.0312	0.0313	0.0087	8.07	55.19
	0.0313 0.0316	0.0315	0.0088	8.11	55.44
16	0.0454 0.0450	0.0452	0.0126	11.67	79.75
	0.0456 0.0456	0.0456	0.0127	11.78	80.51
24	0.0523 0.0524	0.0524	0.0146	13.52	92.40
	0.0520 0.0524	0.0522	0.0145	13.48	92.15

Velocity Constants.

TABLE I*a*
Nucleic Acid.

t	$Mg_2P_2O_7$ (x)	$a-x$	$\frac{1}{t}\log\frac{a}{a-x}$
min.	*gm*		
0			
60	0 0089	0 0481	0.0012
240	0 0308	0 0262	0.0014
	$a = 0\ 0570$		
Average..........			0 0013

TABLE II*a*.
Nucleic Acid.

t	$Mg_2P_2O_7$ (x)	$a-x$	$\frac{1}{t}\log\frac{a}{a-x}$
min.	*gm*		
0			
60	0 0062	0 0367	0.0011
120	0 0140	0 0289	0 0014
240	0 02ᶜ2	0 0177	0.0016
480	0 0365	0 0064	0 0017
960	0 0438		
1,440	0 0462		
	$a = 0\ 0429$		
Average................................			0 0014

TABLE III*a*.
Nucleic Acid.

t	$Mg_2P_2O_7$ (x)	$a-x$	$\frac{1}{t}\log\frac{a}{a-x}$
min.	*gm.*		
0			
60	0 0059	0.0370	0.0011
120	0 0131	0 0298	0.0013
240	0 0254	0.0175	0.0016
480	0 0367	0.0062	0.0017
960	0 0437		
	$a = 0\ 0429$		
Average....................... ..			0.0013

TABLE IVa.

Guanosinphosphoric Acid.

t	$Mg_2P_2O_7$ (x)	$a-x$	$\frac{1}{t}\log\frac{a}{a-x}$
min.	*gm.*		
0			
60	0.0097	0.0433	0.00146
120	0.0199	0.0331	0.00170
240	0.0339	0.0191	0.00185
480	0.0449	0.0081	0.00170
720	0.0488	0.0042	0.00140
960	0.0495	0.0035	0.00188
	$a = 0.0530$		
Average...			0.00167

TABLE Va.

Adenosinphosphoric Acid.

t	$Mg_2P_2O_7$ (x)	$a-x$	$\frac{1}{t}\log\frac{a}{a-x}$
min.	*gm.*		
0			
60	0.0110	0.04674	0.00153
120	0.0224	0.03534	0.00178
240	0.0373	0.02044	0.00188
480	0.0478	0.00994	0.00159
960	0.0537	0.00404	0.00162
1,440	0.0543	0.00344	0.00156
	$a = 0.05774$		
Average...			0.00166

TABLE VIa.

Uridinphosphoric Acid.

t	$Mg_2P_2O_7$ (x)	$a-x$	$\frac{1}{t}\log\frac{a}{a-x}$
min.	*gm.*		
0			
60	0.0030	0.0605	0.00035
120	0.0074	0.0561	0.00044
240	0.0136	0.0499	0.00044
480	0.0258	0.0377	0.00047
960	0.0417	0.0218	0.00048
1,440	0.0513	0.0122	0.00049
	$a = 0.0635$		
Average...			0.00044

TABLE VIIa.
Uridinphosphoric Acid.

t	$Mg_2P_2O_7$	$a-x$	$\frac{1}{t}\log\frac{a}{a-x}$
min.	*gm*		
0			
60	0 0035	0.0549	0.00045
120	0 0073	0 0511	0.00048
240	0 0137	0.0447	0.00048
480	0 0237	0 0347	0.00047
960	0 0410	0 0174	0.00054
	$a = 0\ 0584$		
Average.................................			0.00048

TABLE VIIIa.
Inosinphosphoric Acid.

t	$Mg_2P_2O_7\ (x)$	$a-x$	$\frac{1}{t}\log\frac{a}{a-x}$
min.	*gm.*		
0			
60	0 0034	0 0536	0 00044
120	0 0061	0 0509	0 00041
240	0 0136	0 0434	0 00049
480	0 0217	0 0353	0 00043
960	0 0363	0 0207	0 00046
1,440	0 0459	0 0111	0 00049
	$a = 0\ 0570$		
Average..			0.00047

TABLE IXa.
Hexothymidindiphosphoric Acid.

t	$Mg_2P_2O_7\ (x)$	$a-x$	$\frac{1}{t}\log\frac{a}{a-x}$
min.	*gm.*		
0			
60	0 0053	0.05142	0.000710
120	0 0104	0.04632	0.000733
240	0 0185	0.03822	0.000714
480	0 0314	0 02532	0.000730
960	0 0454	0 01132	0.000717
1,440	0 0522	0 00442	0.000752
	$a = 0.5672$		
Average...............................	....		0.000726

THE ESTIMATION OF AMINOETHANOL AND OF CHOLINE APPEARING ON HYDROLYSIS OF PHOSPHATIDES.

BY P. A LEVENE AND T. INGVALDSEN.

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The method herein described is a modification of the one introduced by Thierfelder and Schulze.[1]

In the original method, the separation of the two bases is based on the difference in the behavior of their hydrochlorides towards calcium oxide. Whereas the hydrochloride of aminoethanol is transformed by calcium oxide into the free base, the salt of choline remains unchanged.

In this particular the present method follows the directions of Thierfelder and Schulze. The difference is in the following.

The extraction of the free aminoethanol is accomplished in the original method by means of ether in a Soxhlet apparatus. This process is time-consuming and presents all the inconveniences of a Soxhlet extraction. We substituted in its stead extraction by means of boiling acetone, which is accomplished very rapidly.

A modification was also introduced for the isolation of choline. In the original process the hydrochloride of the base is extracted with alcohol. From this solution the base is precipitated with mercuric chloride, and this is then transformed into the hydrochloride and as such weighed and identified. However, the hydrochloride does not possess sufficiently desirable properties to encourage its use for the purpose of identification or of quantitative estimation. Hence the method was modified as follows:

The residue from acetone extraction is freed from hydrochloric acid and from lime and the choline is converted into the picrate. This if desired can be converted into the chloroplatinate.

[1] Thierfelder, H., and Schulze, O., *Z. physiol. Chem.*, 1916, xcvi, 296.

The ether-soluble, acetone-insoluble lipoids from egg were freed from white matter, and hydrolyzed by, boiling with 3 per cent sulfuric acid for 8 hours as recommended by MacLean.[2] After removal of the fatty acids by filtration, and the sulfuric acid by precipitation with barium hydroxide, the slightly acid filtrate was concentrated in vacuum, and precipitated with basic lead acetate as long as precipitate formed. The precipitate was filtered off, and the filtrate freed from lead with hydrogen sulfide. After addition of hydrochloric acid, the solution of the bases was repeatedly evaporated with water to remove the acetic acid. The residue was then extracted with absolute alcohol, filtered from inorganic salts, and evaporated. The latter operation was repeated twice. Finally the bases were taken up in water and made up to 25 cc. The solution contained 0.887 gm. of total nitrogen and 0.1836 gm. of amino nitrogen.

Of the above solution, 15 cc. were evaporated on the water bath to a small volume. The syrup was thoroughly mixed with an excess of calcium oxide, and extracted three times with dry acetone by boiling it for a few minutes with 75 cc. of that solvent. The combined filtered acetone extracts were acidified with hydrochloric acid, and evaporated in vacuum. The residue was taken up in water and evaporated to a syrup. The treatment with calcium hydroxide and extraction with smaller (25 cc.) portions of acetone were repeated. The final acetone extracts were allowed to stand in an ice chest and filtered. After acidification with hydrochloric acid, the acetone was removed by evaporation in vacuum, and the residue was made up to 10 cc. with water. The solution contained 0.0865 gm. of total nitrogen and 0.08535 gm. of amino nitrogen. Thus 77.5 per cent of the amino nitrogen had been extracted.

Of the above solution 9 cc. were strongly acidified with hydrochloric acid after which 1.9 gm. of gold chloride were added. On standing in a desiccator over sulfuric acid, large crystals separated. These were filtered off, pressed between filter paper, and allowed to dry. The yield was 1.5675 gm. or 71.3 per cent of the theoretical. The substance melted at 188–190°C., and analyzed as follows:

[2] MacLean, H., *Biochem. J.*, 1915, ix, 364.

0.1647 gm. of substance gave 0.0802 gm. of gold on ignition to constant weight.

	Calculated for C₅H₆ON.HAuCl₄. per cent	Found. per cent
Au	49.17	49.06

When the above compound was recrystallized from dilute hydrochloric acid, it separated as large glistening crystals with smooth surface, softening at 185° and melting at 190–192° (uncorrected). The recrystallized compound analyzed as follows:

0.1010 gm. of substance gave 0.0493 gm. of gold on ignition to constant weight.

	Calculated for C₅H₆ON.HAuCl₄. per cent	Found. per cent
Au	49.17	49.11

The residue from the acetone extract was treated with water and filtered from calcium hydroxide. The hydrochloric acid and the remaining calcium were removed from the solution with silver oxide and carbon dioxide, respectively. The filtrate, freed from hydrochloric acid and silver, was acidified with an alcoholic solution of picric acid, concentrated in vacuum, and allowed to stand in an ice chest. The precipitate was filtered off, and recrystallized from water. Beautiful long needles of choline picrate, softening at 238° and melting at 241–242°C. (uncorrected), were thus obtained. The substance analyzed as follows:

0.1302 gm. was reduced with 2 gm. of zinc dust in the presence of 10 cc. of HCl and a little water. The whole was then digested with sulfuric acid as usual, and distilled after addition of enough NaOH to bring all the zinc into solution. The distillate required for titration 15.57 cc. of 0.1 N acid.

0.4164 gm. of the picrate was extracted with ether in the presence of hydrochloric acid. The ether solution was dried over anhydrous sodium sulfate, filtered, and evaporated. The residue was dissolved in dry ether, filtered, and again evaporated. The residue weighed 0.291 gm.

	Calculated for C₅H₁₄ON.C₆H₃O₇N₃. per cent	Found. per cent
N	16.86	16.75
Picric acid	68.97	69.80

0.7714 gm. of the picrate was extracted with ether as above. The aqueous solution was evaporated and the residue was taken up in

absolute alcohol and precipitated with an alcoholic solution of PtCl₄. The yield of dried precipitate was 0.7065 gm. or 98.7 per cent of the theory. The chloroplatinate decomposed at 243°C. and had the following composition.

0.1050 gm. of substance gave on ignition to constant weight 0.0334 gm. of platinum.

	Calculated for $(C_2H_{14}ONCl)_2PtCl_4$. per cent	Found. per cent
Pt..	31.64	31.80

UNSATURATED LIPOIDS OF THE LIVER.

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The subject of the lipoids of the liver calls for reinvestigation for several reasons. Previous investigators have laid claim to the discovery in this organ of substances which were not found among the lipoids of other organs. Furthermore, none of the phosphatides isolated from this organ has been identified with sufficient rigor.

The substances described as peculiar to liver tissue are jecorin,[1] and heparphosphatide.[2] Other unsaturated lipoids isolated from the liver are lecithin (Baskoff) and cephalin.[3] The relation of the latter two lipoids to lecithin and to cephalin of other organs had not been established.

Regarding the unsaturated lipoids of other organs it is known that on the basis of their solubility they may be classified into three groups.

1. The acetone-soluble lipoids.
2. The acetone-insoluble, but alcohol-soluble.
3. The acetone-insoluble and alcohol-insoluble.

These differences in solubility are referred not to the pure substances but to material obtained by the extraction of the tissues with ether.

In this laboratory,[4] it was found that the first fraction consisted principally of lecithin with small proportions of cephalin which can be readily removed; the second fraction consisted of lecithin containing larger proportions of cephalin; the third consisted of very

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[1] Drechsel, E., *J. prakt. Chem.*, 1886, xxxiii, 425.

[2] Baskoff, A., *Z. physiol. Chem.*, 1908, lvii, 395.

[3] Frank, A., *Biochem. Z.*, 1913, 1, 277. Levene, P. A., and West, C. J., *J Biol. Chem.*, 1916, xxiv, 115.

[4] Levene, P. A., and Komatsu, S., *J. Biol. Chem.*, 1919, xxxix, 91.

small proportions of lecithin and larger proportions of cephalin, but the bulk of the material of this fraction is composed of fragments of the other lipoids.

The present investigation aimed to find whether or not the enumerated three fractions with their general characteristics can be obtained from the liver tissue. The answer obtained was in the affirmative.

The acetone-soluble fraction led to the preparation of perfectly pure lecithin. The acetone-insoluble but alcohol-soluble fraction led to a lecithin and cephalin mixture in which cephalin constituted about 20 per cent of the material. From this fraction also practically pure lecithin could be prepared.

The acetone-insoluble and alcohol-insoluble fraction was found to be a mixture of little lecithin, little cephalin, and of a variety of fragments of these and perhaps of other lipoids.

In this respect, then, the lipoid material obtained from the liver does not differ from that of other organs.

The study of the details of the structure of liver lecithin on one hand, and of the cephalin fraction on the other, however, has brought forward some new facts, which are important not only in reference to the special topic of liver lipoids but to that of the unsaturated lipoids in general.

Liver Lecithin.—The point of interest in connection with this substance lies in the nature of the fatty acids entering into the structure of its molecule. In the literature on this subject there exists utter confusion. A critical review of the subject will be given in a future publication. Here it suffices to note that liver lecithin contains in its molecule two fatty acids: one saturated stearic acid and the other unsaturated of the linolic series. There was no evidence of the presence of oleic acid. This conclusion was reached on the basis of the solubility of the barium salt of the unsaturated acid in ether, and on the basis of the hydrogen and iodine values of the fatty acid and of lecithin.

The hydrogen number (grams of hydrogen absorbed by 100 gm. of substance) of a lecithin containing in its molecule linolic acid is 0.5018.

The number found for our lecithin was 0.5018.

The iodine number for two double bonds is 63.26.

The value we found was 72.73.

For linolic acid the hydrogen number is 1.439.

Our estimations on the unsaturated fatty acids from lecithin gave an average of 1.508.

The calculated iodine value is 180 and we found 210.

Whether or not the acid is linolic or its higher homologue is not absolutely certain. The analytical data obtained on the hydrogenated acid prepared in the course of the present work correspond to that required by theory for the higher homologue with C_{20}. Before a definite conclusion is reached a larger quantity of material is required which will permit a more rigorous purification.

In this respect the lecithins obtained from the acetone-insoluble and from the acetone-soluble fractions are identical. The lecithin referred to in this work contained no free amino nitrogen

Cephalin Fraction.—The point of interest in connection with this fraction is the following. As mentioned before, this fraction represents a mixture of different substances among which lecithin and cephalin are present. The special interest of the present work centers on the cephalin. A sample of cephalin having the elementary composition required by theory for this substance has not been obtained. Levene and West had obtained a sample of hydrogenated cephalin with an elementary composition required by theory. This finding established the correctness of the accepted theory of the structure of cephalin. For biological purposes, however, it still remains of importance to prepare a non-hydrogenated cephalin with a theoretical elementary composition. As yet this object has not been attained, but considerable progress towards this end is reported in this communication. Substances with elementary composition approaching closely the one required by theory and containing between 45 to 70 per cent of cephalin and the remaining part lecithin have been prepared.

On hydrogenation of this material a sample was obtained containing 80 per cent of hydrocephalin. The reduced material on hydrolysis yielded stearic acid and aminoethanol.

In a general way the preparation of this material is as follows: Crude cephalin is dissolved in glacial acetic acid. To this solution 99.5 per cent alcohol is added as long as a precipitate is formed.

The filtrate is concentrated under diminished pressure and the residue is emulsified in water and precipitated by means of acetone. The precipitate is the substance mentioned.

The details of the method are given in the experimental part.

A detailed study of the remaining portion of the cephalin fraction will be presented in a separate publication. The application of this new method of fractionation of the cephalin fraction to material obtained from other organs is in progress in this laboratory.

<div align="center">EXPERIMENTAL.</div>

I. Acetone-Soluble Fraction.

Experiment 1.—400 lbs. of liver were minced, dried, and extracted five times with acetone, followed by an equal number of times with moist ether. The ether extracts were concentrated and precipitated with acetone. The precipitate was dissolved in ether and reprecipitated with acetone, the latter operation being repeated several times. All the acetone-ether solutions thus obtained were united with the original acetone extract, concentrated, allowed to stand at 10°C., and filtered. The filtrate was further concentrated *in vacuo* and poured into a large volume of acetone. On standing at 10°C., a syrupy precipitate formed which was separated by decantation. The acetone solution was again concentrated *in vacuo*. The residue was diluted with a little alcohol and precipitated with an alcoholic solution of cadmium chloride as long as a precipitate formed. After standing over night, the precipitate was filtered, redissolved in ether and water as usual, and reprecipitated with alcohol. The precipitate thus obtained weighed 250 gm. and contained 4.5 per cent of its total nitrogen in the form of amino nitrogen.

200 gm. of the above product were heated with 1.5 liters of toluene, allowed to cool, and centrifuged. A solution, No. 180, and a precipitate, No. 181, were thus obtained. The toluene solution was stirred into 6 liters of ether to which 1 per cent of water had been added. The precipitate, No. 180, was separated and washed with alcohol. The yield was 56 gm. of a substance giving the following analysis.

0.1026 gm. of substance gave on combustion 0.1750 gm. of CO_2, 0.0692 gm. of H_2O, and 0.0240 gm. of ash.

0.1898 gm. of substance used for Kjeldahl nitrogen estimation required for neutralization 1.86 cc. of 0.1 N acid.

0.2848 gm. of substance gave on fusion 0.0314 gm. of $Mg_2P_2O_7$.

0.4746 " " " " " " 0.1340 " " $CdSO_4$.

2 gm. of substance were hydrolyzed for a determination of the nitrogen distribution with 10 per cent HCl, neutralized, and then concentrated to 10 cc.

5 cc. of this solution for Kjeldahl determination required 3.95 cc. of 0.1 N acid for neutralization.

2 cc. of this solution gave no nitrogen gas by a Van Slyke determination.

	Calculated for $C_{14}H_{28}O_9NP.2CdCl_2$. per cent	Found. per cent
C	45.13	46.51
H	7.35	7.55
N	1.19	1.36
P	2.65	3.06
Cd	19.20	15.17
NH$_2$ N	0	0
Total N	100	100

The precipitate, No. 181, mentioned above, dissolved for the most part in 500 cc. of benzene The filtered solution was poured into 95 per cent alcohol and a precipitate, No. 181, was obtained. The yield was 40 gm. of a substance containing 0.5 per cent of its total nitrogen in the form of amino nitrogen.

1.5 gm. of substance were hydrolyzed for a determination of the nitrogen distribution with 10 per cent HCl, neutralized, and then concentrated to 10 cc.

5 cc. of this solution required 3.40 cc. of 0.1 N acid for neutralization in Kjeldahl determination.

2 cc. of this solution in a Van Slyke determination gave 0.02 cc. of N gas, $P = 760$ mm., $T = 23°C$.

Experiment 2.—In a second experiment the precipitation of the concentrated acetone liquors with acetone was omitted. In this case, however, it was more difficult to obtain a fraction free from amino nitrogen. After repeated solution in benzene and precipitation with moist ether, two fractions were obtained. Of these, one consisting of 50 gm., was amino nitrogen-free, and the other, 95 gm., contained 0.5 per cent of its total nitrogen in the form of amino nitrogen.

20 gm. of the lecithin-cadmium chloride compound was freed from cadmium chloride by the method described by Levene and West. The lecithin thus obtained was purified once by MacLean's water-acetone method. Attempts to reduce the product failed. Hence the purification with water and acetone' was repeated four times, after which the precipitate was dissolved in ether and reprecipitated with dry acetone.

The following analytical figures on the unreduced material were obtained.

0.1074 gm. of substance gave on combustion 0.2556 gm. of CO_2, 0.1014 gm. of H_2O, and 0.0102 gm. of ash.

0.1940 gm. of substance used for Kjeldahl nitrogen estimation required for neutralization 2.71 cc. of 0.1 N acid.

0.2910 gm. of substance gave on fusion 0.0373 gm. of $Mg_2P_2O_7$.

	Calculated for $C_{44}H_{80}O_8NP$. per cent	Found. per cent
C	65.75	65.72
H	10.70	10.70
N	1.74	1.97
P	3.86	3.61

0.9733 gm. of substance dissolved in 95 per cent alcohol containing one drop of acetic acid was reduced with hydrogen in the presence of palladium by the method of Paal. The palladium and most of the alcohol were saturated with hydrogen before the introduction of the substance. 59.7 cc. of hydrogen gas at 767 mm. pressure and 21°C. were absorbed (54 cc. were absorbed in 1 hour and 15 minutes, and after 4 hours the figures given above were obtained). Reduced to standard conditions, 59.7 cc. at above temperature and pressure are equivalent to 54.4 cc. or 0.00489 gm. of hydrogen gas.

0.3106 gm. of substance absorbed 0.2259 gm. of iodine when titrated according to the methods of Wijs.

	Calculated for $C_{44}H_{80}O_8NP$.	Found.
Hydrogen number	0.5018	0.5018
Iodine "	63.26	72.73

Fatty Acids of Lecithin.

52 gm. of No. 180 and 38 gm. of No. 181 were combined, powdered, and boiled 8 hours with 700 cc. of 10 per cent hydrochloric acid. After cooling, the cake of fatty acids was filtered off. The acids were repeatedly melted in hot water, allowed to cool, and separated

from the water, until the wash water was neutral to litmus. The washed acids weighed about 42 gm. They were converted into barium salts by the method described by Levene and Meyer,[5] using, however, an aqueous solution of barium hydroxide instead of a methyl alcoholic solution as described in the original method. The barium salts were extracted with ether until practically nothing further was taken up by the solvent. A residue, No. 185, and a solution, No. 186, of barium salts were thus obtained. The barium salts of the saturated acids, No. 185, were decomposed with 10 per cent hydrochloric acid on the water bath, and washed free from inorganic acids. The fatty acids, weighing nearly 21 gm., were converted into lead salts by adding an aqueous solution of lead acetate to a methyl alcoholic solution of the acids, followed by a few drops of ammonia water. The lead salts were filtered and washed with water and acetone. The salts were then dissolved in hot benzene and decomposed with hydrogen sulfide. After removing the lead sulfide, the benzene solution was allowed to stand in an ice chest and a precipitate, No. 185, was obtained. The fatty acids in this fraction melted at 68–68.5°C. (corrected) heated at 5 to 6 seconds per degree. After one recrystallization from benzene, the acid melted at 69–70° (corrected). All melting points recorded in this report were taken at such a rate that 5 to 7 seconds were consumed for each degree rise.

The analysis on No. 185 was as follows:

0.1000 gm. of substance gave on combustion 0.2802 gm. of CO_2 and 0.1170 gm. of H_2O.

1.1546 gm. of substance in a molecular weight determination, dissolved in 10 cc. of toluene and 25 cc. of methyl alcohol, required for neutralization 8.08 cc. of 0.5 N alkali.

	Calculated for $C_{18}H_{36}O_2$. per cent	Found. per cent
C	75.98	76.41
H	12.76	13.09
Molecular weight	284	285

The combined benzene filtrates from the above acid were concentrated, and a small volume of acetone was added. After standing

[5] Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1917, xxxi, 627.

in an ice chest, the fatty acids were filtered off and dried. The acids in this fraction melted at 60°C. (corrected) and had the following composition.

0.1005 gm. of substance gave on combustion 0.2809 gm. of CO_2 and 0.1165 gm. of H_2O.

1.2156 gm. of substance in a molecular weight determination titrated as above required for neutralization 8.82 cc. of 0.5 N NaOH.

	Calculated for $C_{18}H_{36}O_2$. per cent	Found. per cent
C..	75.98	76.22
H..	12.76	12.97
Molecular weight....................................	284	275

These acids were again converted into lead salts, which were suspended in ether to remove any trace of unsaturated acids. The free acid after liberation was recrystallized from 95 per cent alcohol, and it then melted at 66–67°C. (corrected) and analyzed as follows:

0.1000 gm. of substance gave on combustion 0.2784 gm. of CO_2, 0.1164 gm. of H_2O, and no ash.

0.5831 gm. of substance for a molecular weight determination titrated as above required for neutralization 4.20 cc. of 0.5 N NaOH.

	Calculated for $C_{18}H_{36}O_2$. per cent	Found. per cent
C..	75.98	75.95
H..	12.76	13.02
Molecular weight....................................	284	278

This substance was apparently stearic acid containing a small amount of impurities.

The ether solution of the barium salts of the unsaturated acids, No. 186, was decomposed with 10 per cent hydrochloric acid in the presence of benzene and washed free of mineral acid as before. The benezene solution of the fatty acids was filtered, evaporated, and finally the acids were dried in a 90° air bath. 20 gm. of a liquid were thus obtained which remained fluid in the ice chest.

4.9110 gm. of substance, dissolved in 95 per cent alcohol and hydrogenated as previously described, absorbed 1.024 liters of hydrogen at 755.5 mm. pressure and 22°C., or 0.0825 gm. of hydrogen.

0.2386 gm. of substance absorbed 0.5028 gm. of iodine when titrated according to the method of Wijs.

	Calculated for $C_{18}H_{34}O_2$.	Found.
Hydrogen number..............................	1.439	1.679
Iodine "	180	210

100 gm. of the same sample of lecithin cadmium chloride compound from which the free lecithin had been obtained were hydrolyzed as previously described. A sample of the free acids was dissolved in ether and repeatedly washed with water. The ether solution was evaporated and the acids were dried in a steam-heated oven.

0.7961 gm. of the mixed acids was dissolved in 95 per cent alcohol and reduced as previously described. The reduction was nearly completed in 15 minutes. The acids absorbed 75.8 cc. of hydrogen at 762 mm. pressure and 22.6°C., or 0.00611 gm. of hydrogen.

	Calculated for $C_{18}H_{34}O_2$.	Found
Hydrogen number.................................	0.714	0.768

It was found that the lead salt of oleic acid was readily soluble in ether while the barium salt of this acid was almost insoluble in that solvent. Hence the rest of the fatty acids were converted into lead salts and extracted with ether. The unsaturated acids were liberated in the ether solution with hydrochloric acid and washed free of mineral acid, after which the ether solution was evaporated. The fatty acids were converted into barium salts and extracted with ether. Only a small residue remained after this extraction, and this gave only a trace of fatty acids after decomposition with hydrochloric acid.

The extracted barium salts were decomposed with hydrochloric acid in the presence of ether and washed free of mineral acid. The ether solution was evaporated and dried as previously described. A liquid was thus obtained which on hydrogenation gave the following figures.

0.8114 gm. of substance, dissolved in 95 per cent alcohol and reduced by Paal's method, absorbed 185.2 cc. of hydrogen at 760 mm. pressure and 23°C., or 0.00997 gm. of hydrogen.

	Calculated for $C_{18}H_{36}O_2$.	Found.
Hydrogen number.................................	1.439	1.339

The unsaturated acids were hydrogenated by Paal's method, and the reduced acids purified by converting them into lead salts.

Repeated recrystallization from 95 per cent alcohol gave acids which melted at 69–70°C. (corrected). With Kahlbaum's stearic, which had a melting point of 68–69°, it melted at 63°C. The substance had the following composition.

0.1006 gm. of substance gave on combustion 0.2850 gm. of CO_2 and 0.1194 gm. of H_2O.

0.1004 gm. of substance gave on combustion 0.2838 gm. of CO_2 and 0.1184 gm. of H_2O.

1.0718 gm. of substance for a molecular weight determination dissolved in toluene and methyl alcohol required for neutralization 6.72 cc. of 0.5 N NaOH.

	Calculated for $C_{20}H_{40}O_2$. per cent	Found. per cent
C..	76.92	77.25, 77.08
H..	12.82	13.28, 13.19
Molecular weight............................	312	319

Further investigation will be required before a final decision as to the actual composition can be reached.

II. Acetone-Insoluble, Alcohol-Soluble Fraction.

Experiment 3.—A lecithin-cadmium chloride compound almost free from amino nitrogen was obtained from the acetone-insoluble, alcohol-soluble fraction of the lipoids. The solution of the lipoids in alcohol was precipitated with cadmium chloride as previously described, and the precipitate was dissolved in benzene and precipitated with ether saturated with water. The operation was repeated four times. At first a large volume of ether was employed, and it was found necessary to add alcohol to accomplish a satisfactory separation. A yield of 57 gm. of a snow-white substance was thus obtained, which contained 0.42 per cent of its total nitrogen in the form of amino nitrogen.

2 gm. of the substance were hydrolyzed to determine the nitrogen distribution, neutralized, and concentrated to 10 cc.

5 cc. of the solution required for neutralization 4.63 cc. of 0.1 N acid in Kjeldahl determination.

2 cc. of this solution gave 0.02 cc. of N gas in a Van Slyke determination, $P = 755$ mm., $T = 23°C$.

The fatty acids from this fraction were obtained as previously described. The mixed acids had the following characteristics.

1.0025 gm. of substance reduced by Paal's method absorbed 97 cc. of H at 761 mm. pressure and 22.2°C., or 0.00751 gm. of hydrogen.

0.3113 gm. of substance absorbed 0.3217 gm. of iodine when titrated according to the method of Wijs.

	Calculated for $C_{18}H_{34}O_2$.	Found.
Hydrogen number.	0.714	0.750
Iodine "	90	103

The saturated acids after one recrystallization from 95 per cent alcohol melted at 6.85°C. (corrected) and had the following composition.

0.1008 gm. of substance gave on combustion 0.2804 gm. of CO_2 and 0.1160 gm. of H_2O.

0.9066 gm. of substance in a molecular weight determination dissolved in toluene and methyl alcohol required for neutralization 6.40 cc. of 0.5 N NaOH.

	Calculated for $C_{18}H_{36}O_2$. per cent	Found. per cent
C	75.98	75.87
H	12.76	12.88
Molecular weight	284	283

Recrystallized from alcohol, this acid melted at 70–72°C. (corrected). A mixture with Kahlbaum's stearic acid, having a melting point of 68–69°, melted at 68.5°C. (corrected).

The ether solution of the barium salts of the unsaturated acids was evaporated. Attempts to dissolve the residue in pure benzene failed as a gelatinous transparent suspension was formed from which the salts could again be separated by centrifuging. The salts dissolved readily in benzene containing 5 per cent absolute alcohol, from which they were precipitated by pouring the solution into a large volume of absolute alcohol. The substance was dried in vacuum over sulfuric acid, at temperature of boiling water, and had the following barium content.

0.0973 gm. of substance gave on fusion 0.0322 gm. of $BaSO_4$.

	Calculated for $C_{36}H_{66}O_4Ba$. per cent	Found. per cent
Ba	19.76	19.48

After allowing the barium salts of the above acids to stand in a desiccator over sulfuric acid for more than a week, their solubility in ether had decreased, and the free acids obtained by decomposition with hydrochloric acid formed a viscous syrup. Oxidation had evidently taken place for on hydrogenation the following figures were obtained.

0.6275 gm. of substance reduced by Paal's method absorbed 71.3 cc. of H at 760 mm. pressure and 22.8°C. or 0.00571 gm. of hydrogen.

0.3455 gm. of substance absorbed 0.4161 gm. of iodine when titrated according to the method of Wijs.

	Calculated for $C_{18}H_{32}O_2$.	Found.
Hydrogen number...............................	1.439	0.911
Iodine "	180	120

Experiment 4.—Another sample of acetone-insoluble, alcohol-soluble lipoid was purified by water and acetone, dried, dissolved in glacial acetic acid, and poured into a large volume of dry acetone. The mixture was allowed to stand at 0°C. over night, when a small precipitate, No. 68, was obtained. This substance had the following composition.

0.1002 gm. of substance gave on combustion 0.2227 gm. of CO_2, 0.0823 gm. of H_2O, and 0.0107 gm. of ash.

0.1960 gm. of substance used for Kjeldahl nitrogen estimation required for neutralization 3.14 cc. of 0.1 N acid.

0.2940 gm. of substance gave on fusion 0.0377 gm. of $Mg_2P_2O_7$.

' 1 gm. was hydrolyzed in estimating the nitrogen distribution, neutralized, concentrated, and made up to 10 cc.

5 cc. of this solution required 2.92 cc. of 0.1 N acid for neutralization in Kjeldahl determination.

2 cc. of this solution gave on Van Slyke determination 1.48 cc. of N gas; $P = 764$ mm., $T = 21°C$.

Found: C = 61.23, H = 9.44, N = 2.30, P = 3.66 per cent.

$$\frac{NH_3 N}{Total N} = \frac{51.4}{100}$$

The acetic acid-alcohol filtrate from No. 68 was evaporated *in vacuo* to a thick syrup, keeping the temperature as low as possible. The residue was emulsified with water and precipitated with a small volume of acetone. The precipitate was dissolved in ether, reprecipitated by acetone, and dried. This substance had the following composition.

0.1059 gm. of substance gave on combustion 0.2523 gm. of CO_2, 0.0971 gm. of H_2O, and 0.0101 gm. of ash.

0.2882 gm. of substance used for Kjeldahl nitrogen estimation required for neutralization 4.39 cc. of 0.1 N acid.

0.2885 gm. of substance gave on fusion 0.0405 gm. of $Mg_2P_2O_7$.

0.5 gm. was hydrolyzed for an estimation of the nitrogen distribution, neutralized, concentrated, and made up to 10 cc.

5 cc. of this solution required 2.16 cc. of 0.1 N acid for neutralization in Kjeldahl determination.

2 cc. of this solution in a Van Slyke determination gave 0.33 cc. of N gas; $P = 768$ mm., $T = 24°C$.

Found: C = 65.38, H = 10.12, N = 2.17, P = 3.92 per cent.

$$\frac{NH_2\,N}{Total\ N} = \frac{13.7}{100}$$

Experiment 5.—Another sample of crude lecithin, which had not been fractionated with acetic acid-acetone, was united with the substance of the above composition and reduced by the method of Paal. After the palladium was removed, the alcoholic solution was allowed to stand in an ice chest when the lipoids crystallized and were filtered off. 56 gm. of lipoids thus obtained were dissolved in chloroform and poured into several volumes of dry ether. The precipitate, weighing 48 gm., was dissolved in 750 cc. of a mixture of methyl ethyl ketone and chloroform as described by Levene and West.[6] 31 gm. separated when the mixture was allowed to stand at room temperature over night. This substance had the following composition.

0.1046 gm. of substance gave on combustion 0.2544 gm. of CO_2, 0.1040 gm. of H_2O, and 0.0104 gm. of ash.

0.2980 gm. of substance used for Kjeldahl nitrogen estimation required for neutralization 3.54 cc. of 0.1 N acid.

0.2944 gm. of substance gave on fusion 0.0396 gm. of $Mg_2P_2O_7$.

0.5 gm. was hydrolyzed for determination of the nitrogen distribution, neutralized, concentrated, and made up to 10 cc.

5 cc. of this solution required for neutralization 2.06 cc. of 0.1 N acid for a Kjeldahl estimation.

2 cc. of this solution gave in a Van Slyke determination 0.52 cc. of N gas; $P = 773$ mm., $T = 25°C$.

Found: C = 67.27, H = 11.28, N = 1.70, P = 3.79 per cent.

$$\frac{NH_2\,N}{Total\ N} = \frac{25.8}{100}$$

[6] Levene, P. A., and West, C. J., *J. Biol. Chem.*, 1918, xxxv, 285.

The filtrate from the above compound was allowed to stand in the ice chest, when a substance of the following composition separated.

0.1035 gm. of substance gave on combustion 0.2499 gm. of CO_2, 0.0964 gm. of H_2O, and 0.0094 gm. of ash.

0.1950 gm. of substance used for Kjeldahl nitrogen estimation required for neutralization 2.74 cc. of 0.1 N acid.

0.2925 gm. of substance gave on fusion 0.0407 gm. of $Mg_2P_2O_7$.

1 gm. was hydrolyzed for estimation of the nitrogen distribution, neutralized, concentrated, and made up to 10 cc.

5 cc. of this solution required for neutralization 4.72 cc. of 0.1 N acid for Kjeldahl determination.

2 cc. of this solution used for Van Slyke determination gave 0.90 cc. of N gas; $P = 765$ mm., $T = 22°C$.

Found: C $= 66.0$, H $= 10.46$, N $= 1.96$, P $= 3.87$ per cent.

$$\frac{NH_2\,N}{Total\,N} = \frac{19.3}{100}$$

III. Cephalin Fraction.

The lipoids precipitated by pouring their solution in ether into a large volume of alcohol were extracted four times with 95 per cent alcohol at 60°C. for 40 minutes as described by Levene and Komatsu.[4] The residue was dissolved in ether, precipitated with acetone, and dried. This substance, No. 6, had the following composition.

0.0968 gm. of substance on combustion gave 0.2180 gm. of CO_2, 0.0750 gm. of H_2O, and 0.0122 gm. of ash.

0.1906 gm. of substance used for Kjeldahl nitrogen estimation required for neutralization 2.84 cc. of 0.1 N acid.

0.2858 gm. of substance gave on fusion 0.0400 gm. of $Mg_2P_2O_7$.

Found: C $= 63.07$, H $= 8.96$, N $= 2.17$, P $= 4.15$ per cent.

The substance was further fractionated by dissolving it in ether and pouring the solution into a large volume of alcohol. Finally the material was separated into two fractions by dissolving it in a small volume of ether and allowing it to stand at 0° for 24 hours. An ether solution, No. 26, and a precipitate, No. 28, were thus obtained. Both Nos. 26 and 28 were then separately dissolved in ether and precipitated with a small volume of alcohol, this operation being repeated several times. After this treatment No. 26 analyzed as follows:

0.1044 gm. of substance gave on combustion 0.2278 gm. of CO_2, 0.0830 gm. of H_2O, and 0.0148 gm. of ash.

0.3155 gm. of substance used for Kjeldahl nitrogen estimation required for neutralization 3.04 cc. of 0.1 N acid.

0.2895 gm. of substance gave on fusion 0.0400 gm. of $Mg_2P_2O_7$.

1.4 gm. of substance was hydrolyzed to determine the nitrogen distribution, neutralized, concentrated, and made up to 10 cc.

5 cc. of this solution required for neutralization 8.05 cc. of 0.1 N acid in a Kjeldahl determination.

2 cc. of this solution used for a Van Slyke determination gave 1.69 cc. of N gas; $P = 757$ mm., $T = 23°C$.

Found: C = 62.86, H = 9.39, N = 1.86, P = 4.06 per cent.

$$\frac{NH_2\,N}{Total\ N} = \frac{55.2}{100}$$

No. 28 analyzed as follows:

0.2381 gm. of substance used for Kjeldahl nitrogen estimation required for neutralization 2.95 cc. of 0.1 N acid.

0.3000 gm. of substance gave on fusion 0.0514 gm. of $Mg_2P_2O_7$.

1 gm. was hydrolyzed to determine the nitrogen distribution, neutralized, concentrated, and made up to 10 cc.

5 cc. of this solution required for neutralization in a Kjeldahl determination 3.25 cc. of 0.1 N acid.

2 cc. of this solution used for a Van Slyke estimation gave 1.58 cc. of N gas; $P = 768.2$ mm., $T = 25°C$.

Found: N = 1.73, P = 4.77 per cent.

$$\frac{NH_2\,N}{Total\ N} = \frac{48.7}{100}$$

A substance analyzing unusually well was obtained from No. 28 by the following procedure. The material was dissolved in three parts of glacial acetic acid with slight warming. The solution was poured into ten parts of alcohol, the precipitate separating on standing in the cold. This was removed by filtration and the filtrate then concentrated *in vacuo*, without heat, to a thick syrup. Attempts to dissolve the residue in ether and reprecipitate it with acetone failed as the lipoid of this fraction was soluble in acetone in the presence of acetic acid. However, the syrup was emulsified with water and precipitated with a small quantity of acetone as described by MacLean for the purification of lecithin. The precipitate was dissolved in ether and reprecipitated with acetone. This substance had the following composition.

0.1036 gm. of substance gave on combustion 0.2372 gm. of CO_2, 0.0838 gm. of H_2O, and 0.0137 gm. of ash.

0.3519. gm. of substance used for Kjeldahl nitrogen estimation required for neutralization 3.54 cc. of 0.1 N acid.

0.2916 gm. of substance gave on fusion 0.0357 gm. of $Mg_2P_2O_7$.

0.8 gm. was hydrolyzed to determine the nitrogen distribution, neutralized, concentrated, and made up to 10 cc.

5 cc. of this solution required for a Kjeldahl determination 1.56 cc. of 0.1 N acid.

2 cc. of this solution in a Van Slyke estimation gave 1.04 cc. of N gas; $P = 759$ mm., $T = 24°C$.

Found: C = 66.00, H = 9.5, N = 1.487, P = 3.60 per cent.

$$\frac{NH_2 \, N}{Total \, N} = \frac{66.4}{100}$$

It was later demonstrated that the acetone-insoluble material, precipitated from its solution in ether with alcohol at room temperature, was well adapted as a starting material for obtaining a substance of the above composition. The material was simply dissolved in acetic acid and poured into a large volume of dry alcohol. The filtrate was treated as previously described. On samples where the carbon was still low, the process was repeated, thereby increasing the carbon content, while the ratio of the amino nitrogen to total nitrogen remained the same. It was more difficult to obtain this material from crude cephalin which had been purified by the hydrochloric acid method. The analyses of some of these fractions are given in Table I.

Several samples of this material were combined, dissolved in hot absolute alcohol, and placed in an ice chest. The precipitate was separated by filtration. The filtrate was evaporated *in vacuo*, and the residue was dissolved in hot methyl ethyl ketone. On cooling to room temperature a small amount of a white solid separated. This substance, No. 131, had the following composition.

0.1000 gm. of substance gave on combustion 0.2400 gm. of CO_2, 0.0966 gm. of H_2O, and 0.0100 gm. of ash.

0.1894 gm. used for Kjeldahl nitrogen estimation required 3.96 cc. of 0.1 N acid for neutralization.

0.2841 gm. of substance gave on fusion 0.0366 gm. of $Mg_2P_2O_7$.

1 gm. was hydrolyzed to estimate the nitrogen distribution, neutralized, concentrated, and made up to 10 cc.

5 cc. of this solution required for neutralization in a Kjeldahl determination 3.25 cc. of 0.1 N acid.

2 cc. of this solution gave in a Van Slyke determination 0.65 cc. of N gas; $P = 752$ mm., $T = 25°C$.

Found: C = 66.78, H = 11.03, N = 2.93, P = 3.66 per cent.

$$\frac{NH_2 N}{Total\ N} = \frac{19.7}{100}$$

The filtrate from No. 131 was evaporated *in vacuo*. The residue was taken up in 95 per cent alcohol, slightly acidified with acetic acid, and reduced by Paal's method. After hydrogenation had

TABLE I.

No.		C	H	N	P	$\frac{NH_2 N}{Total\ N}$
		per cent	*per cent*	*per cent*	*per cent*	
82		64.42	9.92	1.91	3.80	$\frac{45.3}{100}$
95		65.76	9.83	1.52	3.37	$\frac{60.1}{100}$
97		66.00	10.43	1.79	3.58	$\frac{44.1}{100}$
104		64.93	10.03	2.28	3.64	
122		64.78	8.97			$\frac{55.9}{100}$
217		66.46	10.10	2.36	3.48	$\frac{52.2}{100}$
Calculated: for $C_{44}H_{84}O_9NP$ (lecithin)		65.60	10.79	1.74	3.86	$\frac{00}{100}$
for $C_{42}H_{80}O_8NP$		66.17	10.57	1.88	4.17	$\frac{100}{100}$

proceeded for a time a considerable quantity of solids separated which apparently hindered further catalytic action. The tubes were emptied, the solids were separated by filtration in an ice chest, and the hydrogenation was repeated on the filtrate. The reduced

lipoids thus obtained were dissolved in hot absolute alcohol, filtered from palladium, and allowed to cool to room temperature. The resultant precipitate, No. 132, gave the following analysis.

0.1023 gm. of substance gave on combustion 0.2416 gm. of CO_2, 0.0910 gm. of H_2O, and 0.0113 gm. of ash.

0.1947 gm. used for Kjeldahl nitrogen estimation required for neutralization 2.58 cc. of 0.1 N acid.

0.2948 gm. of substance gave on fusion 0.0406 gm. of $Mg_2P_2O_7$.

1 gm. was hydrolyzed to estimate the nitrogen distribution, neutralized, concentrated, and made up to 10 cc.

5 cc. of this solution required for neutralization in a Kjeldahl determination 3.55 cc. of 0.1 N acid.

1.5 cc. of this solution gave in a Van Slyke determination 1.94 cc. of N gas; $P = 752$ mm., $T = 24°C$.

Found: C = 65.98, H = 10.30, N = 1.89, P = 3.93 per cent.

$$\frac{NH_2\,N}{Total\,N} = \frac{71.8}{100}$$

The filtrate from No. 132 was allowed to stand in an ice chest whereupon a precipitate, No. 137, was obtained. This substance gave the following analysis.

0.1029 gm. of substance gave on combustion 0.2480 gm. of CO_2, 0.0942 gm. of H_2O, and 0.0105 gm. of ash.

0.1944 gm. of substance used for Kjeldahl nitrogen estimation required for neutralization 2.03 cc. of 0.1 N acid.

0.2916 gm. of substance gave on fusion 0.0408 gm. of $Mg_2P_2O_7$.

1 gm. was hydrolyzed to determine the nitrogen distribution, neutralized, concentrated, and made up to 10 cc.

5 cc. of this solution required for neutralization in a Kjeldahl determination 3.45 cc. of 0.1 N acid.

2 cc. of this solution gave in a Van Slyke determination 1.79 cc. of N gas; $P = 750$ mm., $T = 25°$.

Found: C = 66.68, H = 10.36, N = 2.02, P = 3.94 per cent.

$$\frac{NH_2\,N}{Total\,N} = \frac{51.1}{100}$$

The filtrate from No. 137 was evaporated. The residue was dissolved in ether and precipitated with acetone. The precipitate, No. 139, gave the following analysis.

0.0944 gm. of substance gave on combustion 0.2232 gm. of CO_2, 0.0906 gm. of H_2O, and 0.0094 gm. of ash.

0.1798 gm. of substance used for Kjeldahl nitrogen estimation required for neutralization 2.18 cc. of 0.1 N acid.

0.2697 gm. of substance gave on fusion 0.0368 gm. of $Mg_2P_2O_7$.

0.5 gm. was hydrolyzed to determine the nitrogen distribution, neutralized, concentrated, and made up to 10 cc.

5 cc. of this solution required for neutralization in a Kjeldahl determination 1.61 cc. of 0.1 N acid.

1.75 cc. of this solution gave in a Van Slyke determination 0.62 cc. of N gas; $P = 773$ mm., $T = 24°$.

Found: C = 65.28, H = 10.87, N = 1.72, P = 3.84 per cent.

$$\frac{NH_2\,N}{Total\ N} = \frac{44.6}{100}$$

After washing the reduction tubes with hot alcohol a solid remained attached to the walls, which was not easily removed with that solvent. This material was dissolved in a mixture of chloroform and alcohol and filtered. After evaporation of the solvent, the residue was dissolved in hot absolute alcohol, filtered, and allowed to cool to room temperature. The precipitate, No. 142, gave a clear colorless filtrate after hydrolysis with hydrochloric acid, and this solution did not reduce Fehling's solution. No. 142 had the following composition.

0.1040 gm. of substance on combustion gave 0.2472 gm. of CO_2, 0.0970 gm. of H_2O, and 0.0109 gm. of ash.

0.1954 gm. of substance used for Kjeldahl nitrogen estimation required for neutralization 2.28 cc. of 0.1 N acid.

0.2938 gm. of substance gave on fusion 0.0402 gm. of $Mg_2P_2O_7$.

0.5 gm. of substance was hydrolyzed to determine the nitrogen distribution, neutralized, concentrated, and made up to 10 cc.

5 cc. of this solution required for neutralization in a Kjeldahl determination 1.07 cc. of 0.1 N acid.

2 cc. of this solution gave in a Van Slyke determination 0.87 cc. of N gas; $P = 754$ mm., $T = 23°$.

Found: C = 66.05, H = 10.62, N = 1.66, P = 3.87 per cent.

$$\frac{NH_2\,N}{Total\ N} = \frac{80.7}{100}$$

10 gm. of No. 139 were hydrolyzed and the bases and acids were obtained as previously[7] described: In this case, however, the extrac-

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[7] Levene, P. A., and Ingvaldsen, T., *J. Biol. Chem.*, 1920, xliii, 355.

tion with alcohol and acetone was not repeated.   0.035 gm. of amino nitrogen was obtained in the acetone extract, and from this 0.33 gm. of a gold salt was obtained which melted at 188°C. and analyzed as follows:

0.1159 gm. of substance gave 0.0570 gm. of Au on ignition to constant weight.

	Calculated for C_2H_6ON $AuCl_4$. *per cent*	Found. *per cent*
Au...	49.17	49.18

The residue from the acetone extract gave a picrate which melted at 240–241° and had the following nitrogen content.

0.2448 gm. of substance used for a modified Kjeldahl nitrogen estimation required for neutralization 29.3 cc. of 0.1 N acid.

	Calculated for $C_2H_{14}ON$ $C_6H_3O_7N_3$. *per cent*	Found. *per cent*
N...	16 86	16.75

The fatty acids were obtained as previously described. The acids were once recrystallized from acetone, and then from ether. The substance melted at 68–69°C. (corrected) and had the following composition.

0.1012 gm. of substance gave 0.2810 gm. of CO_2, 0.1146 gm. of H_2O, and no ash.

1.0294 gm. dissolved in benzene and methyl alcohol in a molecular weight determination required for neutralization 35.3 cc. of a 0.1 N NaOH solution.

	Calculated for $C_{18}H_{36}O_2$. *per cent*	Found. *per cent*
C	75 98	75.72
H...	12 76	12 67
Molecular weight.................................	284	291

STRUCTURE OF YEAST NUCLEIC ACID.

AMMONIA HYDROLYSIS: ON THE SO CALLED TRINUCLEOTIDE OF THANNHAUSER AND DORFMÜLLER.

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In a previous communication[1] were presented the theoretical arguments which militated against the conception of the structure of yeast nucleic acid formulated by Thannhauser and Dorfmüller.[2]

In order to add weight to the theoretical considerations, it seemed desirable to demonstrate by experiment that the trinucleotide described by these authors was in reality a mixture of mononucleotides.

These authors state: "Levene regards yeast nucleic acid as a tetranucleotide. If this view is correct then on mild hydrolysis one should obtain the four nucleotides." Instead, Thannhauser and Dorfmüller claimed to have isolated uridinphosphoric acid and a trinucleotide.

Hence, if it were shown by experiment that the four mononucleotides were formed under conditions of hydrolysis employed by these authors, then the theory advanced by them would automatically fall. True, the present writer has demonstrated the cleavage of the molecule of yeast nucleic acid into four mononucleotides under conditions of hydrolysis that are much milder than those employed by Thannhauser and Dorfmüller, yet it seemed desirable to prove that following the exact directions of these authors and employing efficient methods of separation one obtains in reality a mixture of mononucleotides and not a trinucleotide.

[1] Levene, P. A., *J. Biol. Chem.*, 1918, xxxiii, 425; 1919, xl, 415.
[2] Thannhauser, J. S., and Dorfmüller, G., *Z. physiol. Chem.*, 1917, c, 121.

The conditions of hydrolysis employed by Thannhauser and Dorfmüller were the following: 50.0 gm. of nucleic acid were taken up in 140.0 cc. of 25 per cent ammonia solution and boiled with reflux condenser for 2 hours. In the experiments herein reported 500.0 gm. of nucleic acid were taken up in 1,500 cc. of 25 per cent ammonia water and heated with reflux condenser for 30 minutes; thus the time of the action of the alkali was reduced to one-quarter of that employed in the original experiment of Thannhauser and Dorfmüller.

From the product of hydrolysis there were isolated in crystalline form the three mononucleotides entering into the structure of the molecule of nucleic acid and guanylic acid thus far obtained only in an amorphous condition.

These results were to be expected on the basis of the experience reported in previous publications, and they further confirm the conclusion regarding the structure of the molecule of yeast nucleic acid expressed by the present writer.

EXPERIMENTAL.

Conditions of hydrolysis were practically those of Thannhauser and Dorfmüller, save for the duration of the experiment. 500.0 gm. of nucleic acid were taken up in 1,500 cc. of 25 per cent ammonia water. The product of hydrolysis was concentrated under diminished pressure and separated into the guanylic and adenylic fractions following the directions of Jones and coworkers.[3]

Each fraction was converted into its brucine salt and these were fractionated by crystallization out of 35 per cent alcohol and out of methyl alcohol. The fractionation of this material did not proceed as smoothly as that of the material described in previous publications. As a guide the analysis of the brucine salts of various fractions was used. But the analytical differences of various fractions were not so characteristic as corresponding fractions of brucine salts, when these were obtained from material hydrolyzed under conditions described in previous papers.

[3] Jones, W., and Richard, A. E., *J. Biol. Chem.*, 1914, xvii, 71. Jones, W., and Germann, H. C., *J. Biol. Chem.*, 1916, xxv, 93.

From the most soluble brucine salts (soluble in methyl alcohol) adenylic acid was obtained. The brucine salts were converted as usual into the lead salts and these into free adenosinphosphoric acid.

The analytical data on the substance are as follows:

0.1002 gm. of the substance gave 0.1190 gm. of CO_2 and 0.0418 gm. of H_2O.

0.2000 gm. of the substance employed for Kjeldahl nitrogen estimation gave 26.93 cc. of 0.1 N acid.

	Calculated for $C_{10}H_{14}N_4PO_7 + H_2O$. per cent	Found. per cent
C	32.86	32.39
H	4.41	4.67
N	19.16	18.85

The optical rotation of the substance was

$$[\alpha]_D^{19} = \frac{-0.84 \times 100}{1 \times 2} = -42.0°$$

The mother liquor for the crystalline adenosinphosphoric acid on concentration gave a crystalline deposit resembling cytosinphosphoric acid. The substance was recrystallized out of a solution of one part of water to three of ethyl alcohol, and analyzed as follows:

0.1096 gm. of the substance gave 0.1360 gm. of CO_2 and 0.467 gm. of H_2O.

0.2000 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 18.87 cc. of 0.1 N acid.

	Calculated for $C_9H_{14}N_3PO_8$. per cent	Found. per cent
C	33.42	33.85
H	4.37	4.67
N	13.00	12.86

The rotation of the substance was

$$[\alpha]_D^{19} = \frac{+0.87 \times 100}{1 \times 2} = +43.5$$

The uridinphosphoric acid was obtained from the most insoluble fraction of brucine salts. The brucine salt was converted into ammonium salts. The sample, however, analyzed for a mixture of

mono- and di-basic ammonium salt. The mother liquor of the ammonium salt was transformed into a lead salt which, dissolved on boiling and on slow cooling, settled out in crystals of the appearance of the lead salt of uridinphosphoric acid.

The salt analyzed as follows:

0.1000 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 3.96 cc. of 0.1 N acid.

	Calculated for $C_9H_{11}N_2PO_9Pb.$ per cent	Found. per cent
N..	5.29	5.54

Guanosinphosphoric acid was obtained only in the gelatinous state, and only because of the small yield the material could not be made to crystallize.

THE INFLUENCE OF HYDROGEN ION CONCENTRATION ON THE INACTIVATION OF PEPSIN SOLUTIONS.

By JOHN H. NORTHROP.

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One of the many factors which must be taken into consideration in any experiments with enzymes is the possible inactivation of the enzyme during the course of the reaction. This factor in the case of pepsin has been suggested by Sörensen[1] as the cause of the displacement of the optimum acidity for the digestion of protein to the acid side during the course of the digestion. He considers that the enzyme is more rapidly destroyed by the weak than by the strong acid. Arrhenius,[2] on the other hand, considers that the decrease in the rate of digestion on the acid side of the optimum hydrogen ion concentration for digestion is due to the more rapid destruction of the enzyme by the strong acid. If this explanation is correct the optimum phenomenon loses much of its significance and becomes a secondary characteristic of enzyme activity comparable to the optimum temperature. The possibility also arises that the peculiar falling off of the rate of digestion during the course of the reaction, at any hydrogen ion concentration, is also due to the destruction of the enzyme.

Several investigations[3] have been made on the stability of pepsin in acid solutions from various points of view but the results are not at all concordant. Much of this variation in results is probably due to the failure to realize the importance of the hydrogen ion concentration rather than the total acid concentration.

[1] Sörensen, S. P. L., *Compt. rend. trav. Lab. Carlsberg*, 1909, viii, 162. Sörensen's experiments were made at 52°. They are therefore not strictly comparable with the present results.

[2] Arrhenius, S., Quantitative laws in biological chemistry, London, 1915, 44.

[3] Biernacki, E., *Z. Biol.*, 1891, xxviii, 49. Grober, J. A., *Arch. Exp. Path. u, Pharmacol.*, 1904, li, 103. Liebmann, P., and Johannesen, L., *Ugesk. Læger*, 1911. lxxiii, 902. Ramsay, C. F., *J. Am. Pharm. Assn.*, 1917, vi, 1047.

In the experiments considered in this paper the effect of the following variables on the inactivation of pepsin in solution has been studied: (1) the hydrogen ion concentration; (2) the anion of the acid; and (3) the purity of the enzyme solution.

The results of the experiments are given in Tables I and II and in Figs. 1 and 2. The figures in the tables are the relative amounts of active enzyme present in the solution after 24 or 48 hours. The total active enzyme present at the beginning of the experiment is taken as 10 units. The time required to cause a constant change

TABLE I.

Influence of the Purity of the Enzyme Solution on the Destruction of Pepsin at Various Hydrogen Ion Concentrations.

pH	Relative amount of pepsin per cc. of solution after 48 hrs. at 38°C.		
	0.25 per cent active pepsin.	2.5 per cent weak pepsin.	1.5 per cent weak pepsin in 3 per cent egg albumin solution.
6.2		1.0	
5.9			4.6
5.5	2.0	7.4	7.2
5.1	10.0		
4.7		8.4	8.2
4.0	10.0		7.9
3.6	9.0	6.8	7.2
2.0		6.3	7.0
1.2	8.0	6.0	6.8
0.6	7.8		
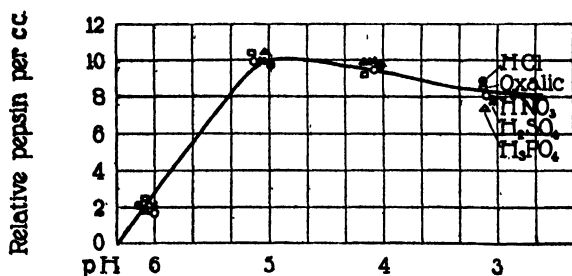
FIG. 1. Relative amount of active pepsin at different hydrogen ion concentrations with various acids after 24 hours at 38°C.

in the conductivity of an egg albumin solution under constant conditions is considered as inversely proportional to the amount of active enzyme present and was used as a measure of the enzyme concentration. The experiments with different enzyme solutions were not done at the same time and are not strictly comparable. The experiments with the various acids, however, are comparable.

It will be seen that in all the experiments the enzyme is most stable at a pH of about 5.0, irrespective of the anion of the acid and of the purity of the solution. Increasing the alkalinity of the solution causes a very great increase in the destruction of the enzyme. There is some indication that the impure solutions are inactivated more slowly under these conditions than the purer ones.

TABLE II.

Influence of Various Acids on the Destruction of Pepsin at Various Hydrogen Ion Concentrations.

pH	Relative amount of pepsin per cc. of solution containing the acids noted below after 24 hrs. at 38°C.				
	HNO$_3$	H$_2$SO$_4$	H$_3$PO$_4$	Oxalic.	HCl
6.0–6.2	1.7	2.1	1.8	2.5	2.2
5.0–5.2	10.0	10.0	10.3	10.0	9.8
4.0–4.2	9.5	10.0	10.0	9.2	9 6
3.0–3.2	8.0	7.9	7.6	8.5	8.8

Increasing the acidity of the solution above pH 5.0 causes a very slow increase in the amount of pepsin destroyed, and the quantity inactivated is not influenced either by the purity of the solution or by the anion of the acid. It would seem necessary to conclude from the marked asymmetry of the curve for the destruction of the enzyme, as plotted against the hydrogen ion concentration, that the process of inactivation of the enzyme on the acid side of pH 5.0 differs from the process of inactivation on the alkaline side of pH 5.0.

Fig. 2 shows that the amount of pepsin remaining in solution after 24 hours at 38°C. is about the same throughout the range of acidity in which the enzyme is active. The rate of destruction of the enzyme therefore differs very little at a pH of 1.0 and a pH of 3.0. As is well known, the activity of the enzyme varies greatly within this range.

This is shown by Curve D, Fig. 2, which is taken from Sörensen's paper and which represents the rate of digestion of egg albumin by pepsin at various hydrogen ion concentrations. If the decline in the rate of digestion on the acid side of pH 2.0 was due to the increased destruction of the pepsin by the acid in greater concentration than this, the same drop should be noticed in Curves A, B, and C as in Curve D, since these curves represent the actual amount of destruction of the enzyme at the acid concentration in question. Fig. 2 shows that this is not the case. But little more enzyme was destroyed at a
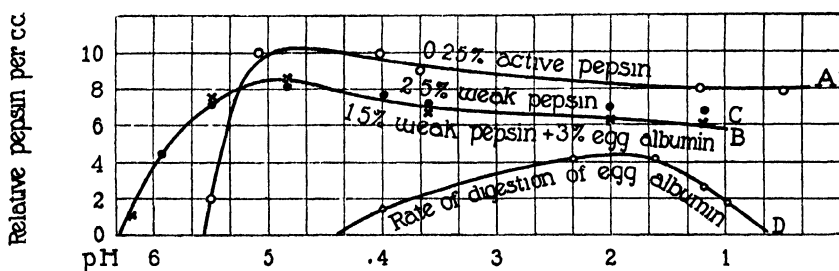
FIG. 2. Relative amount of active pepsin in various solutions at different hydrogen ion concentrations after 24 hours at 38°C.

pH of 1.0 than at a pH of 2.0 or 3.0. The rate of destruction is in any case much too slow to account for the rapid drop in the rate of the digestion curve. This drop is noticeable in the first few minutes of the reaction, while, as the figures show, only 10 to 20 per cent of the enzyme is destroyed in 48 hours at this hydrogen ion concentration.

The fact that the action of the acid on the enzyme is nearly the same across the whole range of hydrogen ion concentration in which the enzyme is active may be considered as indirect evidence that the optimum phenomenon is connected with changes in the substrate rather than in the enzyme. It is apparent from the figures that the enzyme is most stable at a pH of about 5.0; i.e., the same as that for the isoelectric point of many proteins. There is no evidence, however, that pepsin is isoelectric at this point. A series of migration experiments made by the writer confirmed those of Michaelis and Davidsohn[4] (except that the enzyme was never found to migrate to both

[4] Michaelis, L., and Davidsohn, H., *Biochem. Z.*, 1910, xxviii, 1.

poles at the same pH) and gave a change in the direction of migration at about pH 3.0. There is no relation between this point and either the resistance of the enzyme to acid or the rate of its action on proteins. It is probable that this is not the isoelectric point of pepsin itself but that of a compound formed between pepsin and some other substance in the solution, since Peckelharing and Ringer[5] found that very pure pepsin solutions showed no isoelectric point.

No evidence was found that the inactivation of the enzyme was reversible under the conditions of these experiments although many experiments were made with this point in view.[6]

The results show that digestion experiments with pepsin cannot be carried out at 38° for longer than 24 hours without being complicated by the fact that the enzyme concentration is lower at the end of the experiment than at the beginning. They also show that in experiments on the decomposition temperature it is necessary to consider the reaction of the medium.

The general effect of the hydrogen ion concentration on the stability of the enzyme resembles that described by Falk[7] for lipase, and by Frankel[8] for papain. In the case of papain, however, the influence of the reaction is reversed; i.e., papain is more sensitive to acid than to alkali.

Experimental Procedure.

Pepsin Preparations Used.—Active: Fairchild's pepsin u. s. p. 1:19,500 Weak: Pepsin u. s. p. 1:3,000.

Hydrogen Ion Determinations.—All determinations were made by the E. M. F. method.

Determination of the Relative Amount of Pepsin in Solution.

The enzyme solution was made up as shown in the tables and placed in a water bath at 38 ± 0.1°C. 5 cc. of the solution were pipetted out for analysis and 5 cc. of an acid solution added of such strength as to make the final acid concentration in each case equal

[5] Peckelharing, C. A., and Ringer, W. E., *Z. physiol. Chem.*, 1911, lxxv, 282.
[6] Tichomirow, N. P., *Z. physiol. Chem.*, 1908, lv, 107.
[7] Falk, K. G., *J. Biol. Chem.*, 1917, xxxi, 97.
[8] Frankel, E. M., *J. Biol. Chem.*, 1917, xxxi, 201.

to that of the solution containing the highest amount of acid. 1 cc. of this diluted solution was then added to a standard egg albumin solution and the time necessary to cause a 10 per cent change in the conductivity of the latter determined as described in a previous paper.[9] The relative concentration of active pepsin was about the same at the beginning of each experiment. This quantity was taken as 10 in each case. Under the conditions of these experiments neither the products of the digestion of the egg albumin nor the inactivated pepsin interferes with the determination; *i.e.*, the reciprocal of the time to cause a given change is directly proportional to the total quantity of active pepsin present.

SUMMARY.

1. Pepsin in solution at 38°C. is most stable at a hydrogen ion concentration of about 10^{-5} (pH 5.0)

2. Increasing the hydrogen ion concentration above pH 5.0 causes a slow increase in the rate of destruction of pepsin.

3. Decreasing the hydrogen ion concentration below pH 5.0 causes a very rapid increase in the rate of destruction of the enzyme.

4. Neither the purity of the enzyme solution nor the anion of the acid used has any marked effect on the rate of destruction or on the zone of hydrogen ion concentration in which the enzyme is most stable.

5. The existence of an optimum range of hydrogen ion concentration for the digestion of proteins by pepsin cannot be explained by the destruction of the enzyme by either too weak or too strong acid.

[9] Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 113.

THE EFFECT OF THE CONCENTRATION OF ENZYME ON THE RATE OF DIGESTION OF PROTEINS BY PEPSIN.

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The study of the kinetics of enzyme action has led almost invariably to results differing more or less from those predicted by the general laws of chemistry. It would be expected from the general theory of chemical reactions that enzyme reactions should conform to the law expressing the rate of a monomolecular reaction, accelerated by the presence of a catalyst. The rate of reaction, therefore, should be proportional to the concentration of the enzyme and of the substrate and should decrease with time as predicted by the monomolecular formula. This has been found to be true in rare instances[1,2]; but in general the monomolecular formula does not hold for enzyme reactions. It has been found in many cases that the products of reaction interfere with the action of the enzyme. This would account for the divergence of the rate of reaction from that predicted by the monomolecular formula, since, owing to the action of the products, the concentration of the enzyme is changing during the course of the reaction, while the monomolecular formula takes account only of the changes in concentration of the substance decomposed. The rate of reaction of two solutions containing different amounts of enzyme, however, if compared during the same stage of the reaction, should be porportional to the quantity of enzyme, since any effect of the products should be the same in both solutions. It is found in many instances that this is not the case. Enzyme reactions diverge from the expected course of such reactions not only as regards the change in rate with the progress of the reaction, but also in regard to the relation between the rate and the concentration of substrate or enzyme.

[1] Euler, H., *Z. physiol. Chem.*, 1907, li, 213.

[2] Taylor, A. E., *J. Biol. Chem.*, 1906–07, ii, 87. Also Schmitz, H., *J. Gen. Physiol.*, 1919–20, ii, in press.

It was suggested by Brown[3] that these divergences in the case of invertase were due to the fact that the enzyme formed an intermediate compound with the substrate; and several formulas[4] which fit the experimental facts fairly well have been derived on this assumption. They all contain several arbitrary constants, however, and in the lack of any direct evidence in favor of the mechanism which they assume the agreement between calculated and observed values can hardly be considered conclusive. It is assumed in attempting to explain the mechanism of enzyme reactions from the point of view outlined above, that all the enzyme and all the substrate molecules present are equally able to take part in the reaction; in other words, that the active concentration and total concentration of enzyme (or substrate) are the same or directly porportional to each other. It is obvious that, if the active concentration of substrate or enzyme was not equal to the total concentration, the law of mass action would fail to hold if the total concentrations were used in formulas derived from this law, since the law itself states only that the rate of reaction is proportional to the active concentration of the reacting substances. It appears *a priori* quite possible that active enzyme or substrate molecules may exist in solution in equilibrium with other molecules which do not take part in the reaction. The concentration of active enzyme molecules (in the sense of the law of mass action, *i.e.* those which take part in the reaction) would then be some other function of the total concentration and would not be directly proportional to it. The rate of reaction would then also be found to vary as some other function of the total enzyme concentration and not in direct proportion to it. An exactly analogous case is well known in general chemistry; namely, acid hydrolysis.[5] The hydrogen ion is the active part

[3] Brown, A. J., *J. Chem. Soc.*, 1902, lxxxi, 373.

[4] Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1914, xix, 146. These authors review the various other formulas proposed. See also Moore, B., in Hill, L., Recent advances in physiology and biochemistry, New York and London, 1906, 43.

[5] For a general discussion of this question see Stieglitz, J., and collaborators, *Am. Chem. J.*, 1908, xxxix, 29, 166, 402, 650. Stieglitz's experiments were made on the hydrolysis of esters. These solutions can hardly be considered heterogeneous and yet show the same divergences from the simple mass action law as do enzyme reactions. This question will be discussed more fully in a subsequent paper.

of the molecule and the rate of reaction therefore varies directly with the hydrogen ion concentration and not with the total acid concentration. In sufficiently dilute solutions the two of course become practically identical since the acid is then completely dissociated. It will be shown in the succeeding part of this paper that pepsin solutions obey the same laws as weak acid solutions in regard to the relation between the total concentration and the rate of hydrolysis; and that the divergence from the law of mass action is not due to any peculiarity of the enzyme reaction itself, but to the fact that the active enzyme concentration is not always directly proportional to the total enzyme concentration.

Experimental Procedure and Results of the Present Investigation.

In a former paper[6] a method was described for determining the rate of pepsin digestion by means of changes in the conductivity of an egg albumin solution to which the pepsin had been added. From these results the time necessary to cause a given change in the conductivity of the solution was determined by graphic interpolation. In the experiments reported in this paper the time in hours necessary to cause the first 10 per cent change was taken as the standard. The reciprocal of this time then $\left(\dfrac{1}{T \text{ hours}}\right)$ is proportional to the mean rate of digestion for the first 10 per cent of the reaction. For convenience this value will be spoken of as the amount of "active pepsin." The volume noted in the tables is considered in every case as the number of cc. of diluted enzyme solution containing 1 cc. of the original enzyme solution. It is therefore a measure of the dilution of the pepsin before adding to the egg albumin solution. Since 1 cc. of this diluted solution was added to 25 cc. of egg albumin in order to make a determination, the concentration of the pepsin during the actual digestion was $\frac{1}{26}$ of that shown in Tables I, II, III, and V. The conductivity and pH of all solutions were kept equal as nearly as possible. It was pointed out that this change in conductivity did not exactly parallel the change in amino nitrogen of the solution, and so cannot be considered as representing the true course

[6] Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 113.

of the reaction. If the amount of egg albumin and all other factors except the amount of pepsin are kept equal, however, the time necessary to cause a given change may be considered as a definite measure of the rate of reaction, which is all that is necessary for the present purpose.

It was stated[6] that the rate of reaction (*i.e.* the reciprocal of the time to cause a given change) was directly proportional to the concentration of enzyme solution, and that any products of reaction present in the enzyme solution did not interfere with the reaction. Both statements were true as regards the pepsin solutions used in the experiments reported. It was found, however, that some pepsin solutions did not obey this law. The rate of digestion, instead of being directly proportional to the enzyme concentration, increased much more slowly. The same phenomena have been observed by Bayliss in the case of trypsin[7] and invertase,[8] and have frequently been observed in enzyme reactions. It has formed one of the arguments for the conception that the enzyme combines with the substrate according to the adsorption formula.[8,9]

Table I is a summary of an experiment illustrating this point. The results are shown graphically in Fig. 1. It is obvious that the value of *ET* (total pepsin concentration × the time necessary to cause 10 per cent of the total change in conductivity) is constant for low concentrations but increases in higher concentrations. (If the rate of reaction is directly proportional to the enzyme concentration, the value of *ET* must of course be constant.) The calculated figures were obtained by a formula considered below. The key to this behavior is given by the results of the experiments shown in Table II. In this experiment 2.5 cc. of an active pepsin preparation were diluted to 10 cc., A, with HCL (pH 2.0) and, B, with a solution of "peptone"[10] prepared by the digestion of egg albumin by a very small amount of pepsin (but containing no active pepsin). Solutions A and B were

[7] Bayliss, W. M., *Arch. Sc. Biol.*, 1904, ii, suppl., 261.

[8] Bayliss, W. M., *Proc. Roy. Soc. London, Series B*, 1911–12, lxxxiv, 90. Duclaux, E., Chimie Biologique, Paris, 1883.

[9] Bayliss, W. M., The nature of enzyme action, Monograph on Biochemistry, London, New York, Bombay, and Calcutta, 3rd edition, 1914.

[10] The word peptone is used in this paper as a general term for substances with which pepsin combines in solution, but does not hydrolyze.
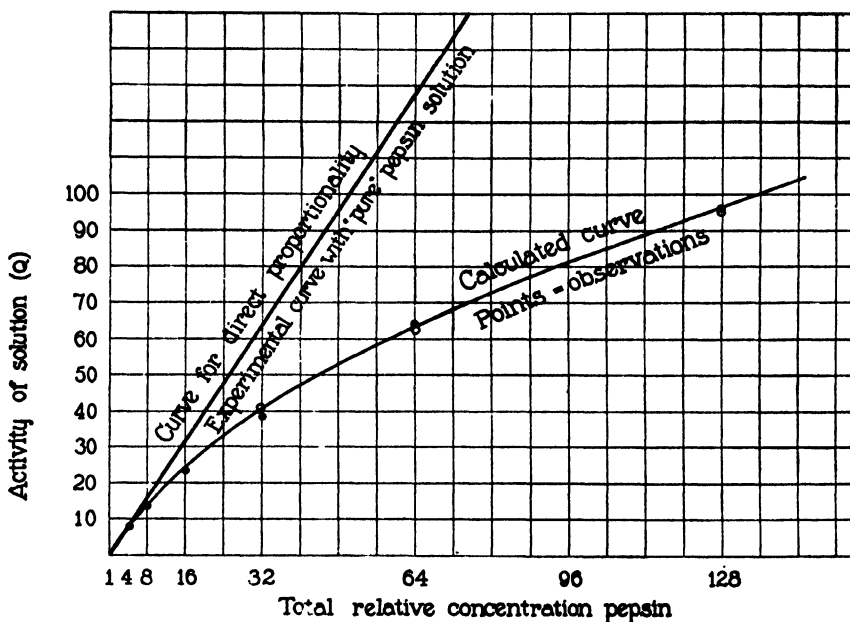
FIG. 1. Curves showing pepsin concentration and rate of digestion (*cf.* Table I).

TABLE I.

Enzyme Concentration and Rate of Digestion.

Pepsin solution.   10 per cent solution of Grübler's pepsin in HCl, pH 2.0.

$$K = 7.2 \qquad d = \frac{30}{v}$$

V = volume containing 1 cc. of original pepsin solution.	E = total pepsin per cc.	$Q = \frac{1}{T}$ = active pepsin per cc.					ET
		Observed.				Calculated.	
		1	2	3	Average.		
1	26.9	9.1	9.7	10.0	9.6	9.7	269
2	13.44	6.25	6.30	6.67	6.39	6.40	206
4	6.72	4.17	3.70	3.57	3.81	4.05	175
8	3.36	2.38	2.50	2.17	2.35	2.42	145 .
16	1.68	1.39	1.43	1.35	1.39	1.38	120
32	0.84	0.83	0.80	0.78	0.80	0.77	106
64	0.42	0.41	0.40	0.39	0.40	0.40	100
128	[0.21]	0.22	0.20	0.20	0.21	0.20	100

then diluted as shown in the table with HCl while Solution C was diluted with Solution B in which the pepsin had been inactivated by making the solution alkaline for 10 minutes.   With Solutions A and C the product of the time into the amount of pepsin present is constant as required by the law of mass action, while in Solution B the value

TABLE II.

Effect of Addition of Peptone to Pepsin Solutions.

Solution A. 2.5 cc. of 2 per cent active pepsin diluted to 10 cc. + HCl, pH 2.0.

Solution B. 2.5 cc. of 2 per cent active pepsin diluted to 10 cc. + 1 per cent peptone solution, pH 2.0.

Solutions A and B then diluted as noted + HCl.   Pepsin determined in 1 cc.

Solution C. Same as B except diluted with inactivated B, instead of HCl.

V = volume containing 1 cc. of original pepsin solution.	E = relative concentration of total pepsin taken.	Time for 10 per cent change in conductivity of 25 cc. of egg albumin + 1 cc. of solution. T = hrs. $\times$ (10²).			ET for solution.		
		A	B	C	A	B	C
1.0	100	20	28	21	20.0	28.0	21.0
1.5	66	31	34	29	20.5	22.5	19.1
2.0	50	40	39	41	20.0	19.5	20.5
4 0	25	83	79	81	20.7	19.8	20.3
8.0	12.5	170	162	178	21.2	20.3	22.2

of the product decreases with increasing dilution until it becomes equal to that value obtained from Solutions A and B, and then remains constant.[11]   The results are plotted in Fig. 2.   The straight line represents direct proportionality.

The solutions were made up to contain the same total concentration of pepsin and in the higher dilutions show the same degree of activity. It seems, therefore, that the divergence of Solution B from the regular law must be due to the fact that the peptone combines with the pep-

[11] This experiment is probably the explanation of the conflicting results obtained by Bayliss[8] and Nelson and Vosburgh (Nelson, J. M., and Vosburgh, W. C., *J. Am. Chem. Soc.*, 1917, xxxix, 790) in connection with the action of invertase.   The activity of the solution of invertase used by Bayliss was not proportional to its concentration whereas the activity of that used by Nelson and Vosburgh was directly proportional.

sin to form a rather highly dissociated compound and that the pepsin so combined is inactive. The concentration of active pepsin would therefore be decreased by the peptone and the decrease would be greater in concentrated than in dilute solution. This hypothesis also accounts for the results of Experiment C in which the solution is diluted with an inactivated portion of the same solution. If the inactivated pepsin enters into equilibrium in the same way as the
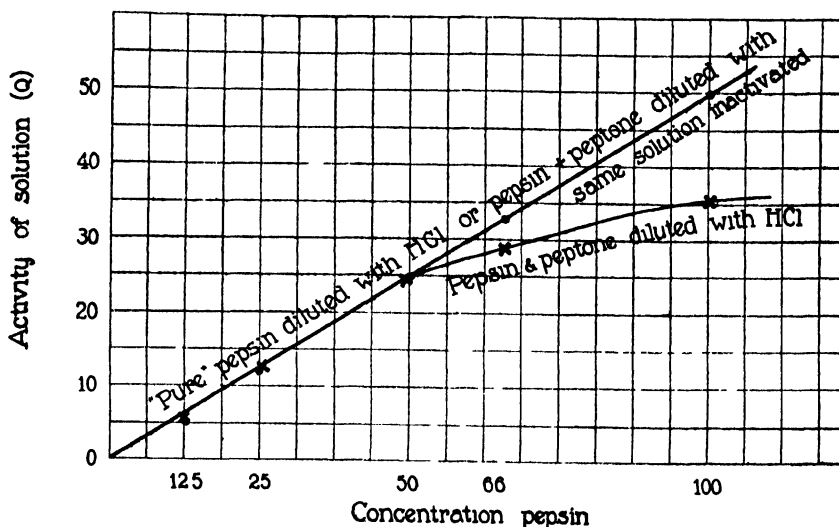
Fig. 2. Curves showing effect of peptone on activity of diluted pepsin solutions (*cf.* Table II).

active pepsin, the concentration of active pepsin in a solution, diluted with an inactivated portion, should decrease in direct proportion to the total concentration. The experiment shows that this is the case. (This question will be taken up more fully later.) The results of this experiment show also that in order to determine the total amount of pepsin present in solution it is necessary to use a dilution such that the rate of digestion is directly proportional to the amount of enzyme solution taken. If this is done the value for the total amount of enzyme, found at dilutions where this value has become constant, is an experimental determination of the total amount of enzyme present, expressed, however, in arbitrary units.

The effect of the peptone in Solution B might be qualitatively explained by the hypothesis that the peptone in the solution combines with the substrate and so reduces the concentration of active substrate molecules, thereby causing the enzyme to become "saturated" with substrate. This explanation, however, fails to explain the results of Experiment C since the same concentration of peptone is present here as in Solution B and yet in this experiment the rate *is* proportional to the amount of enzyme taken.

According to the hypotheses outlined above, the rate of digestion is always directly proportional to the concentration of active pepsin; and the apparent divergence from this relation is due to the fact that the peptone combines with the pepsin and so renders it inactive. The total concentration of enzyme and the active concentration are then no longer equal nor directly proportional; and since the rate is proportional to the active concentration, it is not proportional to the total concentration. It is also assumed that the pepsin and peptone combine according to the law of mass action. This reaction may be considered to take place as follows:

<p style="text-align:center">Pepsin + peptone ⇌ pepsin−peptone</p>

and if the reaction obeys the law of mass action the following equation must hold.

$$\frac{\text{Concentration pepsin} \times \text{concentration peptone}}{\text{Concentration pepsin-peptone}} = K \quad (1)$$

or

$$\frac{Q \cdot (d - (E - Q) + x)}{E - Q} = K \quad (2)$$

where E is the total enzyme concentration, Q is the concentration of active (uncombined) pepsin, d is the concentration of peptone present at the beginning of the reaction, and x is the amount of peptone formed during the course of the reaction at the time t. K is the equilibrium constant expressed in arbitrary units since it contains the unit of measurement used. (For the sake of simplicity only the case is considered in which the substance combined with the pepsin at the beginning of the reaction is the same as that formed during the

digestion.) The value of Q then (the active pepsin concentration) at any moment of the reaction would be that defined by equation (2) or

$$Q = -\frac{d - E + x + K}{2} + \sqrt{\left(\frac{d - E + x + K}{2}\right)^2 + KE} \quad (3)$$

The differential equation for the whole process[12] would then be

$$\frac{dx}{dt} = kQ(A - x) \quad (4)$$

in which Q has the value expressed in equation (3), and A is the active concentration of substrate present at the beginning of the reaction. (It seems quite probable that the active substrate concentration is related to the total substrate concentration in the same way as the active and total enzyme concentrations are related. This question will be discussed later. For the present it is assumed that the rate is proportional to the substrate concentration. At low dilutions of substrate this is an experimental fact.) If this value for Q is substituted in equation (4) it becomes too unwieldy in the integral form to use conveniently. The equation may be tested in the differential form, however, by choosing a small constant value for Δx (taken as 10 per cent of the total change in these experiments) and determining Δt experimentally. The reciprocal of the time necessary to cause the change will then be proportional to the mean rate of reaction during the first 10 per cent of the hydrolysis. This rate is of course decreasing constantly due (1) to the decrease in substrate concentration, and (2) to the decrease in the concentration of active pepsin since some pepsin is removed by combination with the products of reaction. The relative decrease in Q due to (1) is the same in every case and cancels out in comparative experiments, such as are considered here, since the total substrate concentration is kept the same in every experiment. The relative decrease in rate (Q) due to (2), however (as may be seen from equation (2)), will not always be the same but will depend to some extent on the relative values of E, d, and x. It will be shown later that a 5 per cent egg albumin solution when completely digested contains about 10 units of peptone (Table III). The

[12] Neglecting any effect of the reverse reaction.

value of x therefore in the first 10 per cent of the reaction will increase from 0 to 1.0.   The percentage decrease in Q (the concentration of active pepsin) will depend to some extent on the concentration of peptone (d) present at the beginning of the reaction.   That this is actually so is shown by the fact that the relative rates of digestion of two solutions containing the same amount of pepsin but very

TABLE III.

Enzyme Concentration and Rate of Digestion.

Pepsin solution.   1 per cent active pepsin + 10 per cent egg albumin, pH 2.0. Digested 24 hrs. at 37°C.   Diluted as below + HCl, pH 2.0.

$$K = 8.5 \qquad d = \frac{19.2}{v}$$

V = volume containing 1 cc. of pepsin solution.	E = total pepsin.	$Q = \frac{1}{T}$ = active pepsin.			
		Observed.			Calculated.
		1	2	Average.	
1.0	10.95	4.35	4.24	4.29	4.40
1.18	9.32	4.17	4.05	4.11	4.08
1.43	7.66	3.84	3.57	3.70	3.71
1.66	6.58	3.45	3 50	3.47	3.39
2.0	5.48	3.03	2.98	3.00	3.06
2.5	4.39	2.94	2.78	2.86	2.67
3.33	3.28	2.17	2.38	2.27	2.17
5.0	2.18	1.75	1.50	1.62	1.61
10.0	1.09	1.0	0.98	0.99	1.05
20.0	0.54	0.57	0.52	0 55	0.52

different amounts of peptone vary, depending on what stage of the reaction is compared.   This is due to the fact that the rate of digestion of the solution containing the peptone decreases more slowly than that of the solution containing no peptone.   This is in agreement with the formula.   The differences in the percentage decrease in the rates of digestion of two solutions during the first 10 per cent hydrolysis, due to variations in the relative values of E and d, were found to be too small to effect the results within the range of values of E and d used in these experiments.   The relative mean rate for the first 10 per cent hydrolysis may therefore be considered proportional

to the amount of active pepsin present at the beginning of the reaction; *i.e.*,

$$\text{Rate} = \frac{1}{T} = Q = -\frac{d - E + K}{2} + \sqrt{\left(\frac{d - E + K}{2}\right)^2 + KE}$$

where T is the time in hours necessary to complete the first 10 per cent of digestion. This equation may be tested experimentally by testing the constancy of K for various values of E and d or, better, by comparing calculated and observed values of $\frac{1}{T}$ since small experimental errors cause very large changes in the value of K.

The results of such a series of experiments have been given in Table I. Table III contains the results of a similar experiment in which the pepsin solution was prepared by adding 10 per cent of egg albumin to an active pepsin solution and allowing digestion to be completed at a temperature of 38°C. The solution was then diluted as shown in Table III. As was the case in Experiment 1, the rate of digestion is not directly proportional to the total enzyme concentration. It will be seen that in both Tables I and III the agreement between calculated and observed values is within the experimental error. The figure for E, the total enzyme present, is determined directly from the experiments in high dilution when the value of ET has become constant. It was shown in Experiment 2 that the value for E obtained in this way was really proportional to the total amount of enzyme present. The value for d, the amount of peptone present at the beginning of the reaction, is determined from the figures themselves and therefore must be considered as a second arbitrary constant. This fact, of course, detracts considerably from the significance to be attached to the agreement between the observed and calculated values. It will be shown below, however, that under certain conditions the formula may be still further simplified so as to contain one constant and that it is still found to hold.

Table IV contains a summary of an experiment in which the total concentration of peptone was kept the same and the concentration of pepsin increased. The results are shown graphically in Fig. 3. The values for E, the total pepsin present in the solution of Grübler's pepsin, K, the equilibrium constant, and d, the concentration of pep-

tone originally present, were taken from Table I.   The value for E in Solution B was determined by a separate experiment.   It will be seen that the total amount of active pepsin found in the solution is not equal to the sum of the amount of active pepsin added plus the amount of active pepsin already present.   This shows that the pepsin

TABLE IV.

Addition of Active Pepsin Solution to Solution of Grübler's Pepsin.

Solution A. 10 per cent Grübler's pepsin, pH 2.0.

Solution B. 3 per cent active pepsin, $E = 4.2$.

$K = 7.2, d = 3.0, E$ (in Solution A) $= 2.69$ (Table I).   1 cc. of Solution A + noted cc. of B made up to 10 cc. $K = 7.2$.

Volume of Solution B added.	Units of active pepsin added per cc.	Units of active pepsin in Solution A per cc.	$Q = \frac{1}{T} =$ total units of active pepsin.	
			Found.	Calculated.
cc.				
0	0	[2.08]	2.08	2.03
1	0.42	2.08	2.32	2.27
2	0.84	2.08	2.83	2.71
3	1.26	2.08	3.12	3.05
5	2.10	2.08	3.84	3.77
7	2.94	2.08	4.44	4.50
9	3.78	2.08	5.20	5.26

is, in equilibrium with the substance that inhibits its action.   The fact that the calculated values agree with those found by experiment shows that the equilibrium obeys the law of mass action since the calculated figures are obtained by means of this law.

The Effect of Inactivated Pepsin on the Equilibrium.

The results of Experiment 2 show that if a solution of pepsin (A) containing peptone is diluted with acid, the activity of the resulting solution *is not* directly proportional to the concentration of A.   If the same solution is diluted with a portion of itself in which the pepsin has been inactivated with alkali, the activity of the resulting solution *is* directly proportional to the concentration of A.   This is the result predicted if it is assumed that the inactive pepsin enters into the equilibrium (*i.e.* combines with the peptone) to the same extent as

the active pepsin. Table V summarizes the result of an experiment similar to Experiment 2 but covering a wider range. In this experiment an impure solution of pepsin (the same as used in Experiment 1) was diluted, A, with acid of the same hydrogen ion concentration, and, B, with the same solution which had been previously inactivated by alkali and then brought back to the same pH as the original. The
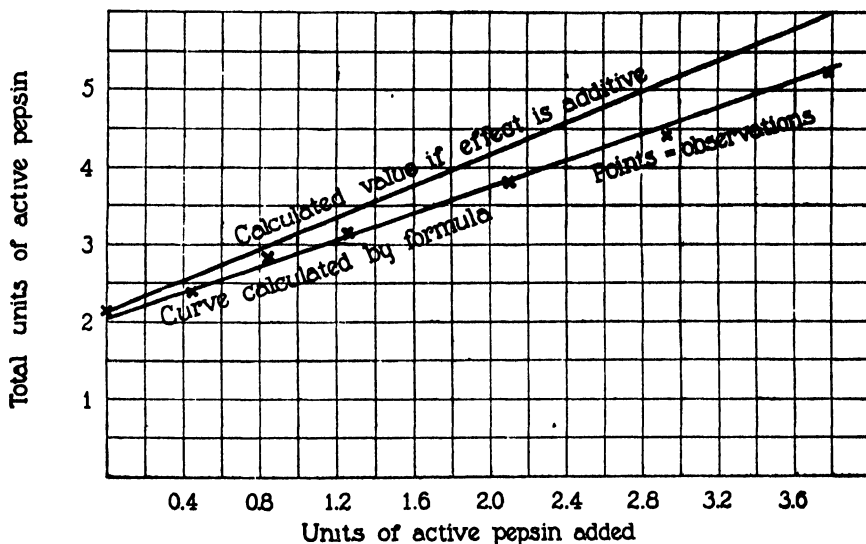
FIG. 3. Curves showing effect of addition of "pure" pepsin to pepsin solution containing peptone (cf. Table IV).

activity of the solution diluted with acid is not directly proportional to its concentration. When the same solution is diluted with an inactivated portion of itself, the activity of the resultant solution is directly proportional to its concentration. The figures show again that the result is predicted quantitatively by the hypothesis. Pepsin inactivated by alkali therefore retains the ability to combine with peptone exactly as does the active pepsin. It has, however, lost the power to hydrolyze protein. A very similar phenomenon is known in immunology—the so called toxoids; i.e., toxins which are no longer injurious but are able to bind antibody in the same way as true toxin.

If the pepsin is inactivated by boiling instead of by treatment with alkali the results become irregular and do not agree with the hypothesis that the inactivated pepsin either does or does not enter into the equilibrium.[18] In order to predict them quantitatively it becomes necessary to assume that either the equilibrium constant is changed

TABLE V.

Influence of Inactivated Pepsin on Equilibrium.

10 per cent Grübler's pepsin diluted as noted with, A, HCl, pH 2.0. B, with same solution inactivated by alkali.

V = volume containing 1 cc. of original pepsin solution.	$Q = \frac{1}{T}$ = active pepsin observed in solution.		Calculated.	
	A	B	If inactive pepsin enters equilibrium.	If inactive pepsin does not enter equilibrium.
1	9.6	9.5	9.6	9.6
2	6.39	5.0	4.8	3.6
4	3.81	2.48	2.40	1.4
8	2.35	1.33	1.20	0.8
16	1.39	0.70	0.60	0.4

or that some of the peptone also is destroyed. In any case boiling causes a different change in the properties of a pepsin solution from inactivation with alkali.

Effect of Adding Increasing Amounts of Peptone to Pepsin Solutions.

It is possible to test further the hypothesis outlined in this paper by noting the effect of adding different amounts of peptone to a constant quantity of pepsin and comparing the observed and calculated activity of the resultant solution. If, as assumed in the hypothesis, the pepsin combines with the peptone to form a dissociated compound, the effect of adding successive equal amounts of peptone to a constant quantity of pepsin should not result in a constant decrease in activity of the solution for each unit of peptone added. The first unit of

[18] An apparently similar phenomenon was noticed by Bayliss[7] in his experiments with trypsin.

TABLE VI.

Effect of Adding Increasing Amounts of Peptone to Pepsin Solution.

0.5 cc. of 5 per cent active pepsin solution + noted cc. of peptone solution (from digested egg albumin) and made up to 10 cc. $K = 8.5$ (Table III), $E = 3.16$, $d = 0.85$ per cc. of peptone solution.

Peptone solution.	d = units of peptone added.	$Q = \frac{1}{T}$ = units of active pepsin.			Units of combined pepsin per unit of peptone added. Observed.	Qd
		Observed.	Average.	Calculated.		
cc.						
0	0	3.03 3 14 3.33	3.16			
1	0 85	2.86 2.86 2.94	2.88	2.92	0.33	2.4
2	1.7	2.70 2.70 2.86	2.75	2.75	0.24	4.6
4	3.4	2.38 2.40 2.50	2.42	2.42	0.22	8.2
6	5.1	2.08 2.04 1.96	2.03	2.13	0.22	10.0
8	6.8	1.67 1.85 1.92	1.81	1.91	0.20	12.0

peptone added should have a greater effect than the second, the second a greater than the third, and so on; the relative decrease of the effect depending on the value of the equilibrium constant. Table VI and Fig. 4 give the result of an experiment carried out in this way. It will be seen that the effect of adding increasing units of peptone agrees very well with the calculated values. The compound pepsin-peptone is widely dissociated at this dilution inasmuch as with a total concentration of 0.85 units of peptone and 3.16 units of pepsin only 0.28 units are combined. This fact is shown graphically in

Fig. 4 where the straight line represents the concentration of active pepsin which would be present if the combination was complete. Table VI also shows that the first unit of peptone inactivates more pepsin than the second, etc.   This phenomenon is also common in immunology and is known as Ehrlich's phenomenon.   As Arrhenius[14] has pointed out it is a general property of any equilibrium system.
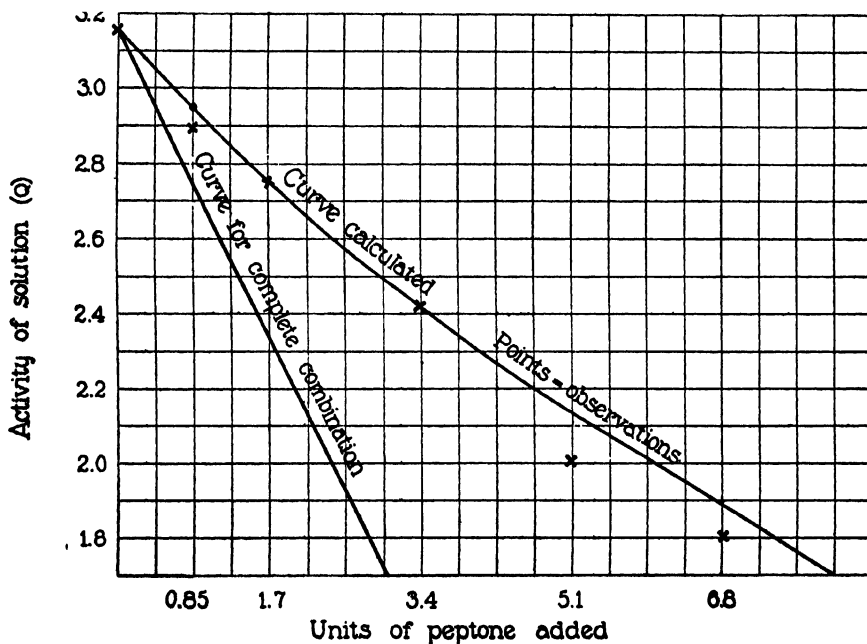
FIG. 4. Curves showing effect of adding increasing amounts of peptone to pepsin solutions (cf. Table VI).

In several other respects the action of pepsin on an egg albumin solution is more or less analogous to the action of toxin on an organism. In a sense the pepsin may be said to make the egg albumin solution immune to pepsin.   That is, if a small amount of pepsin is allowed to act for a long time on a large quantity of albumin it will at first digest it very rapidly and the rate of digestion will be proportional to the amount of pepsin added.   The rate of digestion decreases rapidly,

[14] Arrhenius, S., *Ergebn. Physiol.*, 1908, vii, 480.

however, and finally becomes almost negligible in spite of the fact that there is still a large amount of egg albumin in solution and that the pepsin still retains its activity (as may be demonstrated by diluting the solution, after which digestion will continue). The addition of a further amount of pepsin to the solution will now have little or no effect. The albumin solution is "immune" to the pepsin. This is due to the fact that a small amount of pepsin can cause the production of a very large amount of peptone. Each unit of peptone produced decreases the amount of free pepsin somewhat; but as may be seen from equation (2) it would require an infinite concentration of peptone (d) to reduce the concentration of free pepsin (Q) to 0. Practically, the reaction stops owing to the destruction of the pepsin.[16]

Referring again to Table VI, it will be noted that Qd, the product of the concentration of active enzyme into the concentration of peptone, approaches a constant value as d increases. In other words the concentration of active enzyme becomes nearly inversely proportional to the concentration of peptone, when the latter is present in great excess. This is a well known property of mass action equilibria and follows from the formula, as may be seen below. The formula used in this connection is

$$\frac{Q \cdot [d - (E - Q)]}{E - Q} = K$$

where Q is the concentration of active (free) enzyme, $[d-(E-Q)]$ the concentration of free peptone, and $E-Q$ the concentration of combined pepsin or peptone. It is obvious that as d increases Q must decrease so that the value of the term $E-Q$ approaches the constant value E. When d becomes very large compared with E the term $d-(E-Q)$ will not differ significantly from d. The equation may then be written

$$Q = \frac{KE}{d}$$

d in this equation represents the amount of peptone present at the beginning of the reaction. If the equation is to hold throughout the reaction the concentration of peptone will be represented by $d+x$.

[16] Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 465.

If the simplest case is considered in which there is no peptone present at the beginning of the reaction the concentration of peptone at any time is x (since d is 0) and the formula becomes

$$Q = \frac{KE}{x}$$

This value for Q may now be substituted in equation (4) which becomes

$$\frac{dx}{dt} = \frac{KE(A-x)}{x} \quad (5)$$

in which A is the concentration of substrate at the beginning of the reaction and x is the amount of substrate decomposed (or of peptone formed) at the time T. For the first part of the reaction the value of $(A-x)$ will not differ much from the value of A and the equation may be still further simplified to

$$\frac{dx}{dt} = \frac{KEA}{x}$$

which states that the rate of digestion at any moment is directly proportional to the enzyme concentration and the substrate concentration, and inversely proportional to the amount of substrate decomposed. K in this equation is a new constant equal to the product of k, the velocity constant, and K (equation (4)), the equilibrium constant. This equation, as has been pointed out by Arrhenius,[16] is the differential form of Schütz's[17] rule, since on integration it becomes

$$TKEA = x^2 \quad \text{or} \quad x = K\sqrt{TEA}$$

That is x, the quantity of peptone formed, is proportional to the square root of the time, the concentration of pepsin, and the con-

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[16] Arrhenius, S., *Medd. Kong. vetsakad. Nobelinst.*, 1908, i. An equation similar to this but containing $x\frac{1}{2}$ was found by Bodenstein and Fink (Bodenstein, M., and Fink, C. G., *Z. physik. Chem.*, 1907, lx, 1) to represent the rate of oxidation of SO_2 in the presence of platinum. Dernby, K. G., *Z. physiol. Chem.*, 1914, lxxxix, 425.

[17] Schütz, E., *Z. physiol. Chem.*, 1885, ix, 577.

centration of substrate. It follows from this that if two solutions are compared, each containing the same quantity of substrate, and allowed to digest the same length of time, but with varying concentrations of enzyme, the amount of substrate digested will be proportional to the square root of the enzyme concentration. This is the usual form of Schütz's rule.

It will be remembered that in the derivation of this equation two simplifying assumptions were made: (1) that x, the concentration of peptone, is large compared to Q, the concentration of active pepsin; and (2) that the quantity of substrate present remains relatively constant. The first condition is fulfilled as soon as the digestion has progressed more than a few per cent, provided the original concentration of pepsin is small compared to the concentration of albumin. The second condition, on the other hand, fails to hold after more than 30 or 40 per cent of the substrate is digested. It can be predicted then that Schütz's rule will not hold during the first few minutes of the reaction, or at the end of the reaction, or if the enzyme concentration is too high. As is well known, this is exactly the result obtained by experiment (*cf.* Arrhenius[16]).

The failure of the rule to hold during the first part of the digestion is due to the fact that x at this time is not large compared with Q and hence the relative change in Q is not inversely proportional to the change in x (as assumed in the derivation of the equation) but is much slower as demanded by equation (2). In order to express the fact correctly for the first part of the reaction, then, it would be necessary to substitute for Q in equation (4) the value of Q as defined by equation (3). As has been previously stated, this expression is too unwieldy to handle conveniently. The discrepancy due to changes in the substrate concentration, however, may be corrected very simply if the rate of digestion is directly proportional to the concentration of substrate when the concentration of the latter is low. Experiment shows that this is actually the case. (The effect of the substrate concentration is at present under investigation.) The active concentration of substrate at any moment then will be $A - x$, where A is the original total concentration of substrate and x is the amount transformed. This has already been done in equation (5)

$$\frac{dx}{dt} = \frac{KE\,(A - x)}{x}$$

which on integration becomes

$$\frac{A\ln\dfrac{A}{A - x} - x}{ET} = K \quad (6)$$

If the foregoing hypothesis correctly expresses the mechanism of the reaction, the results calculated from Schütz's rule and equation (6) should agree with the experimental results as soon as x has reached a value ten or fifteen times as large as the quantity of active pepsin present. Before x has reached such a value, the results calculated from equation (6) or Schütz's rule, using the values of K at which they are constant, will be higher than those found by experiment. That is, the value of K in Schütz's rule or equation (6) increases fo· the first 10 or 20 per cent of the total digestion. As was pointed out above, this discrepancy is due to the fact that the formulas are derived on the assumption that the relative change in the pepsin concentration is inversely proportional to the change in the peptone concentration, a condition which does not hold until the peptone is present in large excess. After this point is reached both equations should correctly represent the course of the reaction until the changes in substrate become large. After this change in substrate concentration becomes significant Schütz's rule will no longer hold since there is no term in it that provides for the change in substrate concentration.[18] Equation (6) should hold (*i.e.* give a constant value for K) until the end of the reaction, since this equation takes account of the changes in substrate concentration. Table VII and Fig. 5 give the results of an experiment in which the rate of hydrolysis of an egg

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· [18] If the substrate concentration is high (*i.e.* more than 1 to 2 per cent) Schütz's rule will be found to hold throughout the greater part of the reaction. This is due to the fact that the rate of digestion in concentrated solutions is nearly independent of the substrate concentration. The falling off in the rate of reaction is therefore almost entirely due to the changes in the pepsin concentration. This change is correctly expressed by Schütz's rule. In high concentration of albumin Schütz's rule therefore fits better than Arrhenius' equation.

albumin solution has been followed by means of the conductivity. The total change was $\frac{1}{170}$ reciprocal ohms. The figures have been calculated to the basis of 1,000. The results show that the equations

TABLE VII.
Rate of Hydrolysis.

Substrate = 2.0 per cent egg albumin solution, pH 2.0, = 10.0 units of peptone per cc. when completely hydrolyzed (from Table III).

Pepsin = 0.02 per cent = 0.2 units per cc.

Temperature 38°C. $A = 1,000$.

Time. Hrs. × 10²	Increase in resistance. = X	Values of $K = \dfrac{X}{\sqrt{ET}}$	Values of $K = \dfrac{A \ln \dfrac{A}{A-X} - X}{ET}$
1	1	1	0.06
7	43	16	0.14
11	62	18.7	0.23
18	118	28	0.42
22	143	30	0.51
26	183	36	0.73
31	212	38	0.85
36	240	40	0.94
41	260	40	1.00
51	282	39.5	1.00
61	335	43	1.17
73	360	42	1.17
97	415	42	1.17
125	454	40	1.18
260	582	36	1.12
362	652	34	1.10
462	690	32	1.0
562	740	31	1.1
3,600	1,000		

fit the experimental results almost exactly as was predicted from the derivation. The decline in the value of the constant near the end of the reaction of equation (6) is due to the fact that the changes in conductivity of the solution do not accurately represent the digestion at the end of the experiment.[19] It will be shown later that, when

[19] It has been shown in another paper in this *Journal* (Northrop, J. H., *J. Gen. Physiol.*, 1919-20, ii, 475) that the destruction of pepsin under the conditions of this experiment is so slight as to be negligible. The decrease in the rate of reaction cannot be ascribed to this cause.   .

the changes in digestion are followed by means of the increase in amino nitrogen, which probably accurately follows the digestion, equation (6) gives a constant value for K.

Equation (6) is identical with that derived by Arrhenius[16] from the action of ammonia on a great excess of ethyl acetate, and applied by him to peptic digestion. Arrhenius, however, considered A as representing the concentration of ammonia (which would correspond to the concentration of pepsin in these experiments). In other words
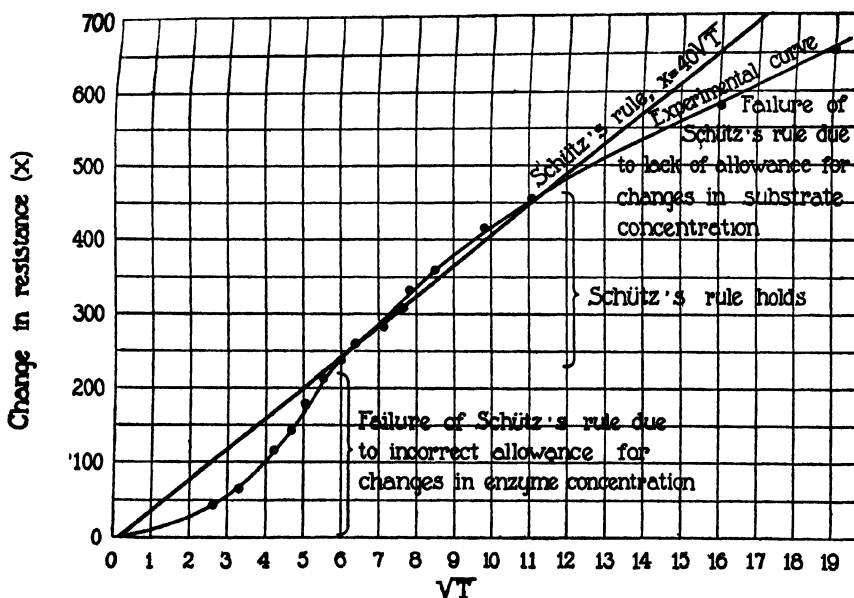
FIG. 5. Curves showing rate of digestion of egg albumin (*cf.* Table VII).

the entire term $\dfrac{(A-x)}{x}$ in Arrhenius' equation represents the ammonia (or enzyme) concentration while the substrate concentration is considered to remain constant. It is clear from the derivation of the equation presented in this paper, however, that the term $\dfrac{E}{x}$ represents the enzyme concentration, while $A-x$ represents the substrate concentration. The equation as applied to the hydrolysis of ethyl

acetate by a small amount of ammonia or to the hydrolysis of protein by pepsin is therefore identical in form but differs as to the significance of the term $A - x$.

In all the foregoing experiments the rate of digestion has been followed by means of changes in the conductivity of the solution. Since this value does not accurately represent the course of digestion, the objection might be raised that the agreement between observed and calculated values is due to compensating errors in the derivation of the equation and in the deviation of the conductivity changes from the actual progress of digestion. This could not be the case in the tests of the equation in the differential form since in this case the results are comparative and any deviation of the conductivity changes from the true rate would cancel out. It is possible, however, that the agreement of the equation in the integral form might be due to some such compensation of errors. In order to show that this is not the case a series of experiments was made in which the course of digestion was followed by means of the increase in amino nitrogen. This value was determined by Van Slyke's[20] method as already described,[21] and, as far as is known, accurately represents the progress of digestion. In these experiments the quantity of egg albumin was kept constant (0.5 per cent egg albumin) and the concentration of pepsin varied. The results are summarized in Table VIII. The figures given under X are the increase in amino nitrogen in cc. per 660 cc. of solution. They are the average of three determinations and have an experimental error of about 10 per cent. This is sufficient to account for the variations in the constant of equation (6). The errors in x are greatly magnified in this constant as it depends on the difference between two experimental values.[22] In every case sufficient time had elapsed before the first observation so that x at the time this determination was made was already large compared to the concentration of pepsin. The change in value of the constants for the first minutes of the re-

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[20] Van Slyke, D. D., *J. Biol. Chem.*, 1912, xii, 275.

[21] Northrop, J. H., *J. Gen. Physiol.*, 1918–19, i, 607.

[22] The errors in x are reduced in the constant of Schütz's rule. The variations in this constant are therefore outside the limits of experimental error while those of Arrhenius' constant are within the limits of experimental error.

TABLE VIII.

Comparison of Schütz's Rule and Arrhenius' Equation.   Rate of Hydrolysis.

Substrate = 0.5 per cent egg albumin solution, pH 2.0.

X = relative increase in amino nitrogen per cc. of solution.

E = relative pepsin concentration.

Time.	X	E	$K = \dfrac{X}{\sqrt{ET}}$	$K = \dfrac{\left[A \ln \dfrac{A}{A-X} - X\right]}{ET}$
min.				
30	380	32	12.0	0.10
75	500	32	10.0	0.08
135	640	32	10.0	0.08
255	700	32	8.5	0.05
495	806	32	6.6	0.05
840	860	32	5.0	0.04
1,740	975	32	4.0	0.05
4,200	[1,000]	32		
30	224	16	10.0	0.06
75	382	16	12.0	0.07
135	528	16	11.0	0.10
255	608	16	10.0	0.07
495	620	16	7.0	0.04
840	700	16	6.0	0.04
1,740	860	16	5.0	0.04
4,200	[1,000]	16		
30	152	8	10.0	0.06
75	277	8	11.0	0.08
135	390	8	12.0	0.10
255	516	8	11.0	0.10
495	536	8	12.0	0.06
840	656	8	8.0	0.05
1,740	742	8	6.5	0.06
4,200	930	8	2.3	0.07
30	100	4	13.0	0.045
75	174	4	10.0	0.055
135	225	4	11.0	0.055
255	363	4	11.0	0.08
495	415	4	9.4	0.06
840	490	4	8.5	0.055
1,740	636	4	7.6	0.055
4,200	770	4	6.0	0.045

TABLE·VIII—*Concluded.*

Time.	x	E	$K = \dfrac{x}{\sqrt{ET}}$	$K = \dfrac{\left[A \ln \dfrac{A}{A-x} - x \right]}{ET}$
min.				
30	90	2	11.5	0.07
75	139	2	11.5	0.07
135	180	2	11.0	0.07
255	265	2	12.0	0.06
495	305	2	10.0	0.06
840	385	2	9.5	0.06
1,740	500	2	8.5	0.06
4,200	630	2	7.0	0.05
30	80	1	14.5	0.10
75	93	1	11.0	0.06
135	112	1	10.0	0.05
255	165	1	10.0	0.06
495	180	1	8.0	0.03
840	230	1	8.0	0.04
1,740	350	1	8.5	0.05
4,200	420	1	6.5	0.03

action, noticed in Table VII, is therefore lacking here. The results show that the equation gives a fairly satisfactory constant when it is considered that the experimental observations are very difficult and that the experiments represent changes in the value of E, x, and T of many hundred per cent. Individual experiments were made which gave much more constant values for K. The present series is given preference, however, since it shows that the equation takes into consideration changes in the enzyme concentration. It is obvious, however, that this equation is merely an approximation formula which will hold only under certain limited conditions and is but little more general than Schütz's rule. The derivation given offers a rational interpretation of both expressions. It may be pointed out also that equation (6) contains only one arbitrary constant K and can therefore hardly be considered as empirical.

DISCUSSION.

It has been shown in the preceding paragraphs that the divergence of the kinetics of pepsin action, from the results predicted from the

law of mass action, may be quantitatively explained by the assumption that the enzyme in solution is in equilibrium with the products of digestion of the protein, or some other substance, and that this equilibrium obeys the ordinary laws of mass action. The results of Peckelharing and Ringer[23] may be taken as experimental proof that the enzyme is so combined. These authors found that very pure solutions of pepsin showed no isoelectric point when tested between two oppositely charged electrodes; but that the addition of peptone caused the pepsin to change the direction of migration at a pH of about 3.0; which corresponds approximately to the isoelectric point of these added substances. It is difficult to explain this experiment otherwise than to conclude that the pepsin combines with the peptone and is carried with it to the electrode. If some of the pepsin combines with peptone, therefore, and so becomes inactive the rate of digestion will evidently not be directly proportional to the total concentration of pepsin but to some other function of the total concentration as defined by the mass action equilibrium. This is exactly analogous to the relation between the hydrogen ion concentration and the total acid concentration. In this case it is only the hydrogen ion which is active in hydrolysis and the activity of the solution is therefore not directly proportional to the total acid concentration. (In the case of acid it is known that the dissociation is electrolytic; *i.e.*, the dissociated parts of the molecule are electrically charged. Whether this is true or not in the case of the pepsin cannot be stated as yet.) The rate of reaction then becomes directly proportional to the active (free) enzyme concentration as demanded by the law of mass action; and the apparent divergence from this law is due to the fact that the total enzyme concentration and the active enzyme concentration are not always directly proportional; just as the total acid concentration and the active acid concentration are not always directly proportional. If this hypothesis is correct, it seems probable that the enzyme does not combine with the substrate for an appreciable length of time, but that the contact of enzyme and substrate molecule results in immediate decomposition of the latter into its products of digestion. There is no doubt that the enzyme actually does combine with the substrate when the latter is not in solution.[6] It is quite possible, however, that this is a case of solution of the en-

[23] Peckelharing, C. A., and Ringer, W. E., *Z. physiol. Chem.*, 1911, lxxv, 282.

zyme in the solid phase and that the kinetics of the reaction are the same there as in the liquid phase.   There is some experimental evidence in favor of this point of view.   It was found that the rate of digestion of edestin was the same when in solution and when suspended in the enzyme solution.[21]   Dauwe[24] has shown that pepsin can diffuse through a membrane of solid protein.

The hypothesis enables us to set an upper limit for the purity of an enzyme preparation.   It was found, for instance, in Experiment 1, that the enzyme solution used contained about twenty-seven (arbitrary) units of pepsin and about thirty units of peptone.   Assuming that the combining weights of the substances are approximately the same it is obvious that the original preparation could not have been more than 50 per cent pure pepsin.   It is, however, quite possible that the enzyme may be combined with some substance and still retain its activity (as found for invertase combined with charcoal by Nelson and Griffin[25]) or that impurities are present which do not combine with the enzyme at all.   It is not possible therefore to assume that the active pepsin consists solely of pepsin molecules.   For similar reasons it is not possible to draw any definite conclusions from the results of Experiment 2 in which it was found that a 1 per cent egg albumin solution after complete digestion contained about two arbitrary units of peptone while a 1 per cent pepsin solution contained about ten units of pepsin.

It is well known that the kinetics of enzyme reactions differ in another respect from the general laws of chemical reactions in that the rate of reaction in high concentration of substrate does not vary directly with the total substrate concentration.   This phenomenon is very similar to the one discussed in the present paper and it would seem that the same explanation applies to both cases; *i.e.*, that the active substrate concentration is not directly proportional to the total substrate concentration.

It may be pointed out that, according to the above mechanism of the reaction, pepsin cannot be considered a catalyst in the sense of the classical definition since it combines with some, at least, of the products of reaction and so enters directly into the equation.   Since

[24] Dauwe, F., *Beitr. Chem. Physiol. u. Path.*, 1905, vi, 426.
[25] Nelson, J. M., and Griffin, E. G., *J. Am. Chem. Soc.*, 1916, xxxviii, 1109.

the enzyme combines with one (at least) of the products of reaction its presence must necessarily affect the equilibrium point. The reaction, therefore, would appear to be a special case of bimolecular reaction in which one of the reacting substances (the enzyme) forms a highly dissociated compound with one of the products. The truth of the matter probably is that so called pure catalytic reactions are merely limiting cases in which the combination of the catalyst is so small as to escape detection (Stieglitz).[5]

SUMMARY.

1. In certain cases the rate of digestion of proteins by pepsin is not proportional to the total concentration of pepsin.

2. It is suggested that this is due to the fact that the enzyme in solution is in equilibrium with another substance (called peptone for convenience) and that the equilibrium is quantitatively expressed by the law of mass action, according to the following equation.

$$\frac{\text{Concentration pepsin} \times \text{concentration peptone}}{\text{Concentration pepsin-peptone}} = K$$

It is assumed that only the uncombined pepsin affects the hydrolysis of the protein.

3. The hypothesis has been put in the form of a differential equation and found to agree quantitatively with the experimental results when the concentration of pepsin, peptone, or both is varied.

4. Pepsin inactivated with alkali enters the equilibrium to the same extent as active pepsin.

5. Under certain conditions (concentration of peptone large with respect to pepsin, and concentration of substrate relatively constant) the relative change in the amount of active pepsin is inversely proportional to the concentration of peptone and the equation simplifies to Schütz's rule.

6. An integral equation is obtained which holds for the entire course of the digestion (except for the first few minutes) with varying enzyme concentration. This equation is identical in form with the one derived by Arrhenius[16] for the action of ammonia on ethyl acetate.

7. It is pointed out that there are many analogies between the action of pepsin on albumin solutions and the action of toxins on an organism.

ON THE CAUSE OF THE INFLUENCE OF IONS ON THE RATE OF DIFFUSION OF WATER THROUGH COLLODION MEMBRANES. II.

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I.

In this note it is intended to complete the proof that the influence of the concentration of electrolytes on the transport of water through a collodion membrane is similar in the case of free and of electrical endosmose.

Fig. 1 gives the curves representing the influence of different concentrations of KCl, K_2SO_4, K_3 citrate, and $CaCl_2$ upon the initial rate of diffusion of water from pure water through a collodion membrane into solution (free osmosis). The solution was inside a collodion bag and the latter dipped into a beaker filled with distilled water. The ordinates of the curves give the rise in the level of liquid after 20 minutes in a glass tube pushed through a rubber stopper into the collodion flask. The curves show, as pointed out in a preceding publication,[1] that the level rises at first with increasing concentration until it reaches a maximum at about $M/512$ or $M/256$ and that it then drops with a further rise in concentration until the latter is $M/16$ when a second rise begins. The second rise is presumably the expression of a prevalence of the gas pressure effect while the first rise and fall are the effect of the electrostatic influence of the ions on the rate of diffusion of water. As stated in the preceding publication, pure water as well as water containing electrolytes is positively charged when in contact with a collodion membrane while the latter is negatively charged. Leaving aside the gas pressure effect on the rate of diffusion, water will be driven through the pores or interstices of the

[1] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 173.

membrane from that side of the membrane which has a smaller density of charge to that side which has a greater density. The density of charge on a collodion membrane in contact with a watery solution is influenced in an opposite sense by the oppositely charged ions of an electrolyte in solution, the density being increased by that ion which

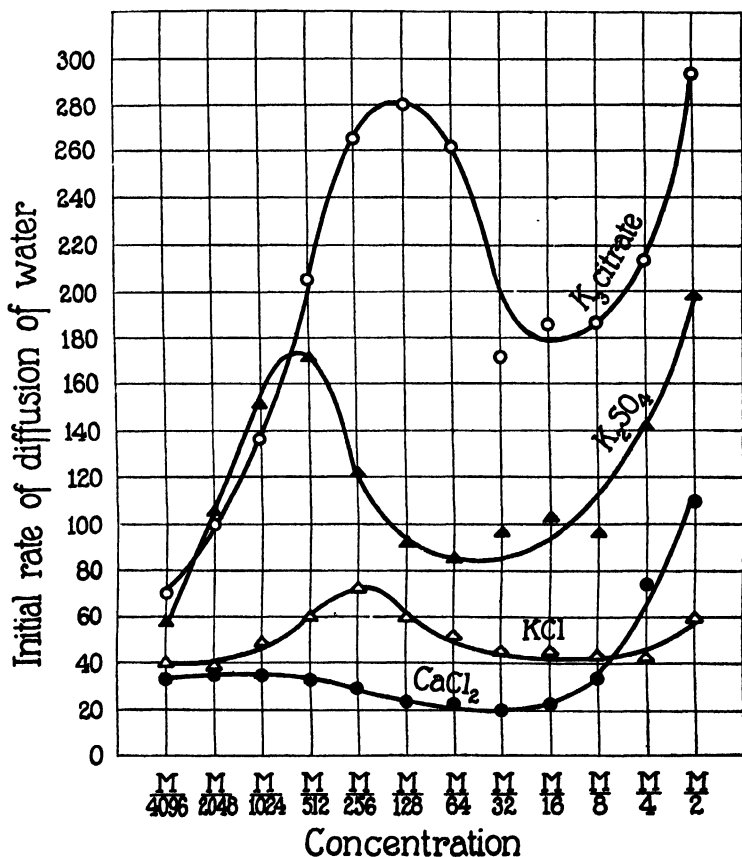FIG. 1. *Free osmosis*. Effect of ions on free osmosis when water is positively charged. Inside the collodion bag, solutions of CaCl₂, KCl, K₂SO₄, and K₃ citrate in different concentration; outside, H₂O. Abscissæ are the logarithms of concentrations of electrolyte in the collodion bag, ordinates the rise in level of liquid in manometer after 20 minutes (with the exception of K₃ citrate which is given after 10 minutes).

has the same sign of charge as the membrane and being diminished by that ion which has the opposite sign of charge.[2] Since the collodion membrane is negatively charged in contact with the solutions mentioned in Fig. 1, the density of charge on the membrane is increased by the anion and diminished by the cation of the electrolyte.

The relative influence of the oppositely charged ions of an electrolyte is, however, *not* the same for different concentrations.   When the membrane is negatively charged the influence of the anion on the charge of the membrane increases in lower concentrations more rapidly with increasing concentration than the depressing effect of the cation until the concentration is $M/512$ or $M/256$.   When this point is reached, the depressing influence of the cation on the negative charge of the membrane increases more rapidly with increasing concentration than the influence of the anion.

This explains why the curves representing the initial rate of diffusion of water from pure water through a collodion membrane to a solution of an electrolyte rise at first with increasing concentration until the concentration is about $M/512$ or $M/256$ and then generally drop with a further increase in concentration.[3]   The rise of the curves is higher the higher the valency of the anion and less the higher the valency of the cation.   In the case of $CaCl_2$ the strong depressing effect of Ca prevents a rise by the Cl ion.

It follows from the theoretical discussion in the preceding paper[2] that if this explanation of the influence of the concentration of the electrolyte is correct, the rate of diffusion of water through a collodion membrane must vary in the same sense with the concentration of the electrolyte in the case of *electrical* endosmose as it does in the case of *free* osmosis.   This is shown to be true by the curves in Fig. 2. These curves represent the relative transport of water in the case of electrical endosmose through a collodion membrane in the presence of the same electrolytes as those used in Fig. 1.

'   In these experiments the collodion flasks were filled with a solution of an electrolyte and were dipped into a beaker containing an identical solution.   A large disc of platinum at the bottom of the beaker

[2] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 387.
[3] The second rise beginning with $M/16$ is due to the gas pressure effect and will not be discussed in this paper.

was one electrode while the other electrode was a platinum wire
pushed through a glass tube into the collodion flask.   The latter was
closed with a rubber stopper through which the glass tube with a
bore of 2 mm. diameter was pushed into the collodion flask.   The
distance between the two electrodes was 7.0 cm.   A P.D. of 15 volts
was applied and the current through the collodion membrane in-
creased slowly in intensity until it finally became fairly constant.
To accelerate this process a P.D. of 200 volts was used for 30 seconds
or a little longer, and then the P.D. was changed to 15 volts.   This,
however, was done only in the case of the more dilute solutions.   The
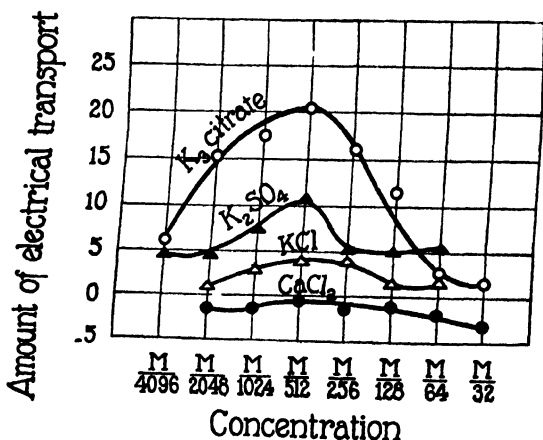
FIG. 2. *Electrical endosmose.*   Effect of the same electrolytes as in Fig. 1 on
electrical endosmose.   Inside and outside the collodion bag identical solutions.
Abscissæ are the logarithms of concentrations of electrolyte in solution, ordi-
nates rise of level of liquid on the side of the cathode in 15 minutes.   The influ-
ence of electrolytes on the rate of transport of water in electrical endosmose is
similar to that in free osmosis (Fig. 1).

liquid rose in the glass tube when it contained the cathode, showing
that the watery phase was positively charged.   The rise in level in the
glass tube was measured for 15 minutes (usually between 5 and 20
minutes after the commencement of the action of the current).
    The reader will notice that the amount of liquid transported by the
current rises at first with the increase in concentration of the solution
until the latter is about M/512 and that with a further increase in

concentration the quantity of electro-endosmotic transport falls. The curves resemble those in Fig. 1 except that the maximum is a little lower in the case of electrical endosmose. The writer is inclined to explain this difference by the fact that in electrical endosmose the concentration of the liquid is increased by the secondary chemical reactions at the electrodes and that thereby in a $M/512$ solution of K_2SO_4 or KCl the concentration of the solution rises gradually as a consequence of electrolysis and secondary chemical reactions at the electrodes and approaches $M/256$.

It is, moreover, obvious that the electrical transport (Fig. 2) of the positively charged liquid increases with the valency of the anion as in free osmosis (Fig. 1) and that it diminishes with the increasing valency of the cation as shown by the flat curve for $CaCl_2$. The curves for $MgCl_2$ and $BaCl_2$ are like those for $CaCl_2$ in the case of free osmosis as well as in the case of electrical endosmose.

We have inferred in the preceding paper[2] that the rate of transport of liquid in electrical endosmose varies, if the other conditions remain equal, with the value of the charge ϵ on the unit area of membrane. If this inference is correct then it follows from the nature of the curves in Fig. 2 that beginning with the lowest concentrations the influence of the anion on the density of charge of the membrane increases at first more rapidly with increasing concentration than the depressing effect of the cation upon the density of this charge, while later the reverse occurs. The turning point seems to lie for the solutions mentioned between $M/512$ and $M/256$, where it also lies for free osmosis.

II.

When we separate solutions of electrolytes with a hydrogen ion concentration of 10^{-4} N or above from pure water by collodion membranes which have been treated with a protein, the watery phase of the double layer is negatively and the membrane positively charged.[1,4] In the preceding paper[2] it was shown that in such a case the charge of the membrane is increased by the cation and diminished by the anion of an electrolyte in solution, both effects increasing with the valency

[4] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 717.
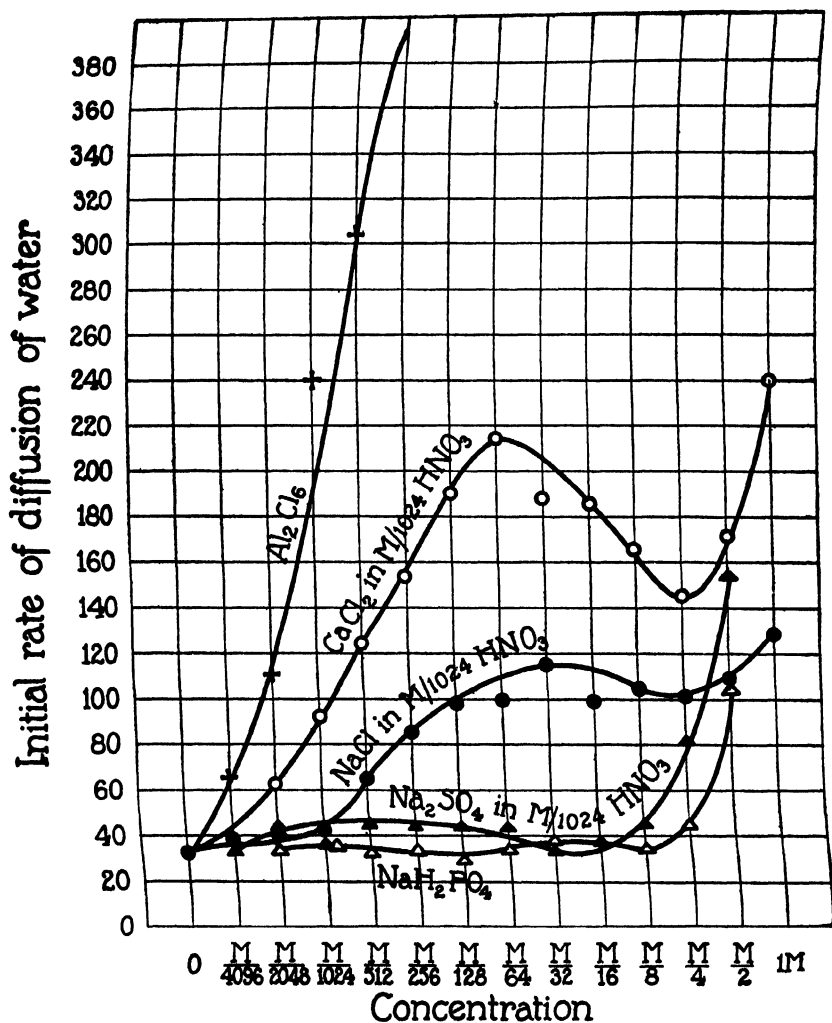
Fig. 3. *Free osmosis.* Effect of ions on free osmosis when water is negatively charged due to acid reaction of solution. Inside the collodion bag acidulated solutions of salts; outside distilled water. Abscissæ are the logarithms of concentration of solution, ordinates rise of level of liquid in manometer after 20 minutes.

of the ion.   Fig. 3 represents the influence of different concentrations of electrolytes (with hydrogen ion concentration above 10^{-4} N) upon the rate of diffusion of water through the membrane.   It increases with the valency of the cation and diminishes with the valency of the anion, the curves in the case of Na_2SO_4 or NaH_2PO_4 showing no rise. The curves for $CaCl_2$ and $NaCl$ show a rise and then a drop, the turning point, however, lying at higher concentrations than for neutral solutions.   The rise and drop find their explanation on the assumption that the influence of the cation on the charge of the membrane
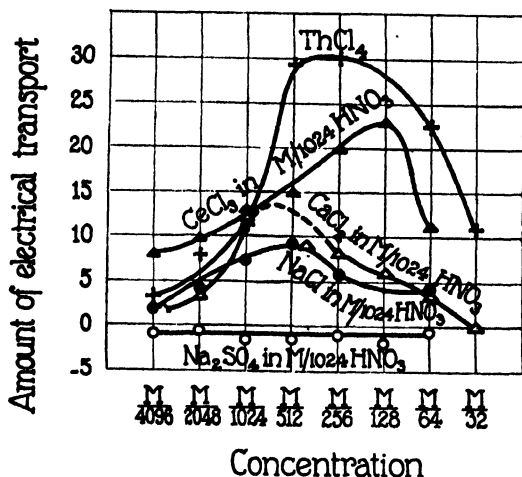
FIG. 4. *Electrical endosmose.*   Effect of acidulated salt solutions on electrical endosmose.   Inside and outside the bag indentical solutions.   Similarity of curves of transport in Fig. 4 and Fig. 3.

increases at first more rapidly with increasing concentrations than the depressing effect of the anion upon this charge, while at concentrations above a certain point the reverse happens.

This conception is supported by experiments on electrical endosmose as represented in Fig. 4.   The solutions of Na_2SO_4, $NaCl$, $CaCl_2$, and $CeCl_3$ were made up in M/1,024 HNO_3 and the hydrogen ion concentration was in the neighborhood of 10^{-3} N.   The $ThCl_4$ solution was sufficiently acid on account of hydrolysis (its pH varying between 3.5 and 1.9 according to concentration).   The watery phase was negatively charged and it was necessary to put the anode into the glass

tube in order to bring about a rise in the level of water in the tube. It is obvious from Fig. 4 that the electro-endosmotic transport of the negatively charged liquid rises at first with increasing concentration of the electrolyte and then falls again; and the initial rise increases with increasing valency of the cation and diminishes with increasing valency of the anion. The turning point varies for different electrolytes, probably on account of secondary chemical reactions at the electrodes, especially acid formation at the anode. The curves if interpreted on the basis of Helmholtz's theory prove that when the membrane is positively charged its positive charge is raised in low concentrations of electrolytes more considerably by the cation than it is depressed by the anion of the electrolyte, while when the concentration of the electrolyte exceeds a certain limit the depressing effect of the anion increases more rapidly with further increase in concentration than the opposite effect of the cation; thus supporting the explanation offered for the phenomena of free osmosis in Fig. 3.

III.

Fig. 5 is a repetition of a figure published in a preceding paper[5] showing the difference of influence of Al_2Cl_6 on the rate of diffusion of water when the collodion membrane has been treated with a protein and when it has not been treated. When the membrane has been treated with a protein, water is powerfully attracted by a solution of Al_2Cl_6 and the attraction increases with the concentration (upper curve); when the membrane has not been treated with a protein, water is not attracted by the solution except when the concentration becomes so high that the gas pressure effect of the solution shows itself (lower curve). The explanation offered by the writer was as follows. When we separate Al_2Cl_6 solutions of sufficiently low concentration from pure water by a membrane treated with gelatin, the water diffuses through the membrane in the form of negatively charged particles which are attracted powerfully by the Al ion and repelled weakly by the Cl ion; or, in other words, the Al ion increases the positive charge of the membrane considerably and the Cl ion depresses the same charge less. As a consequence the density of the positive charge of the membrane on the solution side of the membrane must

[5] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 255.
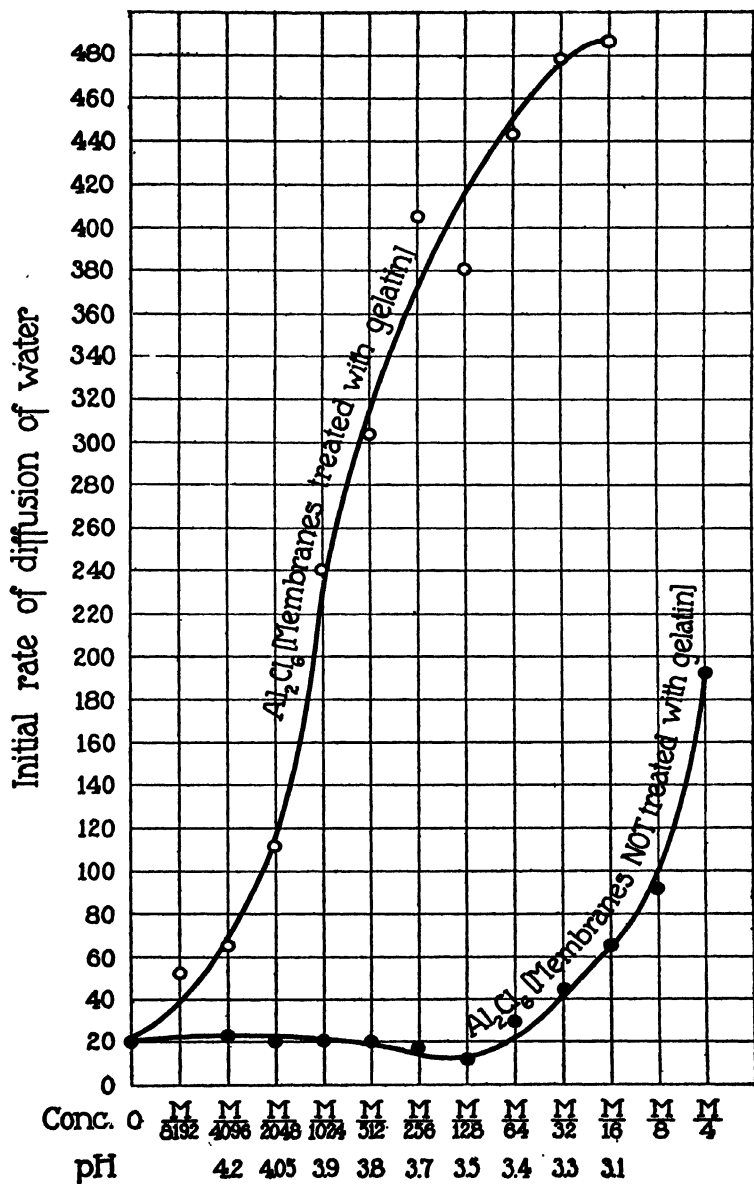
FIG. 5. *Free osmosis.* Upper curve, influence of Al₂Cl₆ upon the rate of diffusion of water when the collodion membrane has been treated with gelatin; lower curve, influence of Al₂Cl₆ on rate of diffusion when the membrane has not been treated with gelatin.

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be greater than the positive charge of the membrane on the side of pure water and water will be driven to the solution side. When, however, the collodion membrane has not been treated with gelatin it is negatively charged even in the presence of Al_2Cl_6 and of acid, and in the presence of the Al ions the Cl ions cannot raise the negative charge on the solution side of the membrane beyond that on the water
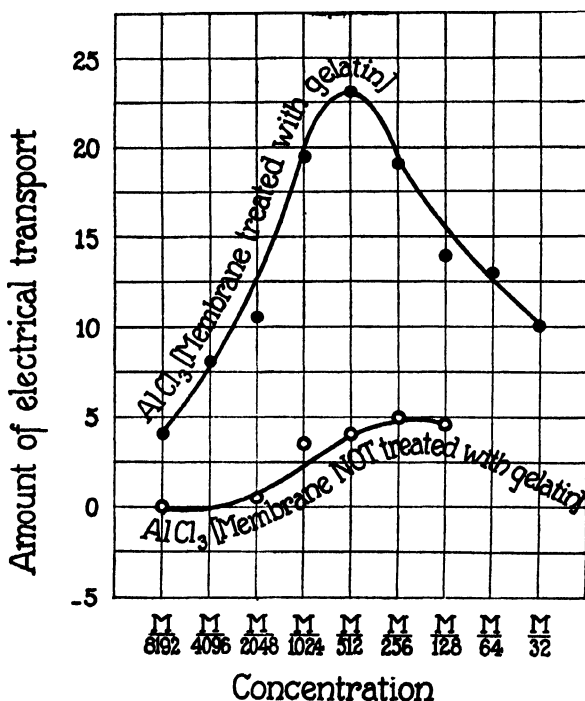
FIG. 6. *Electrical endosmose* with the same two kinds of membrane.

side. As a consequence in this case no other attraction of water by the solution except that based on the gas pressure effect can be produced. If this view is correct, the curves of electro-endosmotic transport of liquid through membranes treated and not treated with gelatin must be similar to the curves of free osmosis in Fig. 5. Fig. 6 shows that this is the case. When identical solutions of $AlCl_3$ are put into the collodion flask and into the beaker into which the collodion flask is dipped, and when a P.D. is applied in the way described,

a transport of water to the anode occurs which increases with increasing concentration to about M/512 provided the membrane had been treated with a protein (upper curve, Fig. 6); when the collodion membrane has not been treated with a protein no such migration occurs (lower curve, Fig. 6). Hence the curves for electro-endosmotic transport of liquid through collodion membranes and the transport of water in free osmosis run parallel, supporting the explanation offered.

In comparing the upper curves in Figs. 5 and 6, the reader will notice a difference, inasmuch as in the case of free osmosis through membranes treated with gelatin (upper curve, Fig. 5) the curve shows only a rise but no drop, while in the upper curve for electro-endosmotic transport (Fig. 6) through a gelatin-treated membrane in the presence of a solution of $AlCl_3$ there occurs the characteristic drop at a concentration beyond M/512. The writer is inclined to attribute the reason for this difference to the fact that in free osmosis the gas pressure effect prevents the drop while in electrical endosmose this gas pressure effect is excluded (since the solutions on both sides of the membrane are identical).

<center>IV.</center>

When we separate a M/256 solution of Na_2SO_4 or Li_2SO_4 by a collodion membrane from pure water, the latter will diffuse into the solution with a certain velocity. When we add small and identical quantities of a salt like KCl to the solution of M/256 Na_2SO_4 and to the distilled water, this velocity will be diminished;[6] owing to the fact that beyond a concentration of M/256 Na_2SO_4 the repelling or depressing effect of the cation of the solute increases more rapidly with increasing concentration of the electrolyte than the attracting or accelerating effect of the anion on the rate of diffusion. When we add $MgCl_2$ or $CaCl_2$ instead of KCl the depressing effect is still greater than in the case of KCl, owing to the fact that Mg and Ca, as bivalent ions, have a greater depressing effect than K. Fig. 7 illustrates this statement, showing that the addition of KCl or $MgCl_2$ or $CaCl_2$ to M/256 Na_2SO_4 diminishes the rate of diffusion of liquid into the Na_2SO_4 solution and that $MgCl_2$ and $CaCl_2$ act more power-

[6] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 273.

fully than KCl. The addition of 0.2 cc. of M/4 MgCl₂ or CaCl₂ to 100 cc. of M/256 Na₂SO₄ (concentration of MgCl₂ or CaCl₂ = M/2,000) lowers the rate of diffusion of water into the solution more than 50 per cent, while the effect of the addition of 0.2 cc. of M/4 KCl (concentration of KCl = M/2,000) lowers the rate of diffusion of water into the solution considerably less.
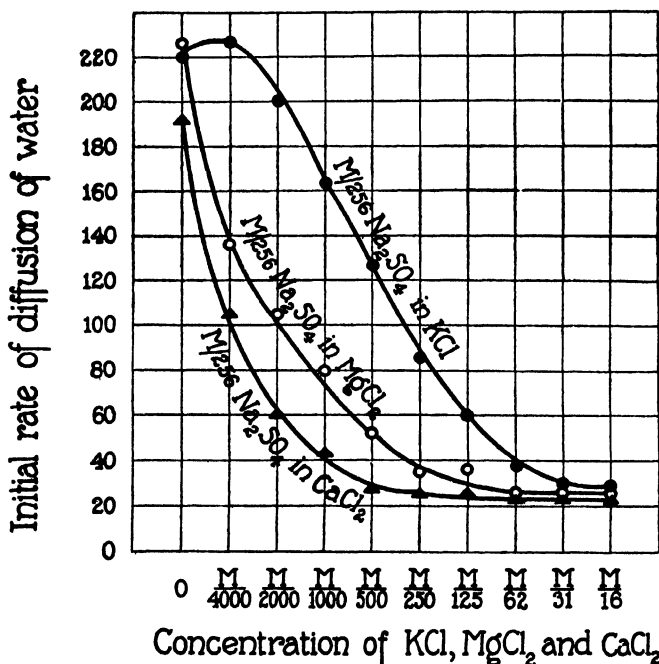
FIG. 7. *Free osmosis.* Depressing effect of the addition of KCl, MgCl₂, and CaCl₂ upon the attraction of water by M/256 Na₂SO₄.

Fig. 8 shows that the addition of KCl and CaCl₂ to M/512 Na₂SO₄ has the same depressing effect on the rate of transport of liquid through the collodion membrane in the case of electrical endosmose. In this, as in the preceding experiments on electrical endosmose, identical solutions were put into both sides of the collodion membrane. These solutions were M/512 Na₂SO₄ alone or with the addition of small quantities of KCl or CaCl₂. A P.D. of 15 volts was produced on the opposite sides of the membrane and the rate of transport of water

(which took place to the cathode) was observed. The curves in Fig. 8 show the result. The quantity of transport of liquid was depressed both by the addition of KCl and of CaCl₂ but more by CaCl₂ than by KCl. The addition of 0.1 cc. of M/4 CaCl₂ to 100 cc. of M/512 Na₂SO₄ (concentration of CaCl₂ = M/4,000) lowers the transport of water more than twice as much as the addition of 0.1 cc. of M/4 KCl. The depressing influence of the addition of KCl or CaCl₂ in the electrical transport of water through a collodion membrane can be demonstrated equally well with M/256 solutions of Na₂SO₄ as with M/512 solutions.
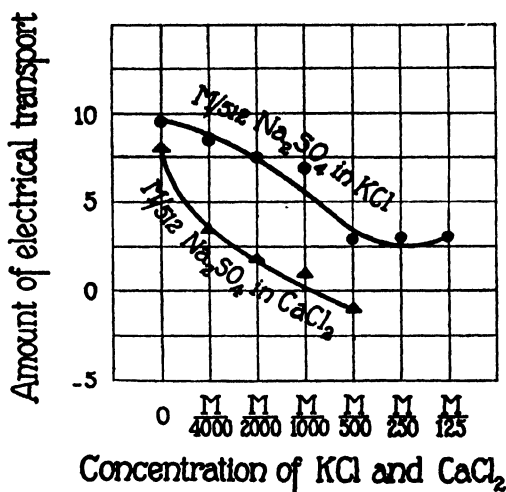
FIG. 8. *Electrical endosmose.* Showing that a similar depressing effect as in Fig. 7 exists in the case of electrical endosmose.

These experiments on electrical endosmose show that the density of negative charge on the membrane in contact with a M/256 or M/512 Na₂SO₄ solution is diminished by the addition of any electrolyte (whose anion does not act more powerfully than SO₄) and that the diminution increases with increase in the valency of the cation added. This furnishes the explanation of the fact that when M/256 Na₂SO₄ is separated from pure water by .a collodion membrane the rate of diffusion of the positively charged water into the solution is diminished by the addition of KCl and still more by the addition of MgCl₂ or CaCl₂.

The writer has duplicated the majority of the experiments he has thus far published on free osmosis by experiments on electrical endosmose and there is a fair degree of similarity in the two cases.

SUMMARY.

1. It had been shown in previous publications that when pure water is separated from a solution of an electrolyte by a collodion membrane the ion with the same sign of charge as the membrane increases and the ion with the opposite sign of charge as the membrane diminishes the rate of diffusion of water into the solution; but that the relative influence of the oppositely charged ions upon the rate of diffusion of water through the membrane is not the same for different concentrations. Beginning with the lowest concentrations of electrolytes the attractive influence of that ion which has the same sign of charge as the collodion membrane upon the oppositely charged water increases more rapidly with increasing concentration of the electrolyte than the repelling effect of the ion possessing the opposite sign of charge as the membrane. When the concentration exceeds a certain critical value the repelling influence of the latter ion upon the water increases more rapidly with a further increase in the concentration of the electrolyte than the attractive influence of the ion having the same sign of charge as the membrane.

2. It is shown in this paper that the influence of the concentration of electrolytes on the rate of transport of water through collodion membranes in electrical endosmose is similar to that in the case of free osmosis.

3. On the basis of the Helmholtz theory of electrical double layers this seems to indicate that the influence of an electrolyte on the rate of diffusion of water through a collodion membrane in the case of free osmosis is due to the fact that the ion possessing the same sign of charge as the membrane increases the density of charge of the latter while the ion with the opposite sign diminishes the density of charge of the membrane. The relative influence of the oppositely charged ions on the density of charge of the membrane is not the same in all concentrations. The influence of the ion with the same sign of charge increases in the lowest concentrations more rapidly with increasing concentration than the influence of the ion with the opposite sign of charge, while for somewhat higher concentrations the reverse is true.

THE REVERSAL OF THE SIGN OF THE CHARGE OF MEMBRANES BY HYDROGEN IONS.

By JACQUES LOEB.

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I. INTRODUCTION.

The similarity between the influence of ions on free and on electrical endosmose through collodion membranes leads to the conclusion that this influence is due to an alteration of the density of the electrical charge of the membrane by the ions.[1] This conclusion rests on the assumption that the Helmholtzian theory of the double layer and the formula connecting the volume of transport of liquid with the density of charge is correct. We have seen that on this assumption the observations lead to the conclusion that the ions with the same sign of charge as the membrane increase and the ions with the opposite sign of charge diminish the density of charge of the membrane. Whether, therefore, a given ion increases or diminishes the density of charge of the membrane depends upon the sign of the charge of the membrane, and, hence, the sign of the charge precedes the influence of ions on the density of charge. One of the problems in the theory of the double layer at the boundary of membrane and watery solution is therefore the origin of the sign of the charge of membranes in contact with water.

Chemists consider the double layer at the boundary of membrane and water as ionic in character and as due to the preferential adsorption of one kind of ions by the membrane, while the watery phase of the double layer is formed by the ions with the opposite charge from those adsorbed. This view has been developed with remarkable lucidity and consistency by Perrin[2] who assumes that the H and OH·

[1] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 387, 563.

[2] Perrin, J., *J. chim. physique*, 1904, ii, 601; 1905, iii, 50; Notice sur les titres et travaux scientifiques de M. Jean Perrin, Paris, 1918.

ions are especially influential in determining the sign of charge of the membrane. In alkaline solutions the OH ions are said to be adsorbed by the membrane, while the mobile, watery stratum of the double layer is formed by the cations; in acid solutions the H ions are thought to be adsorbed while the anions form the mobile stratum of the double layer. When the OH ions are adsorbed by the membrane the latter is negatively charged, when the H ions are adsorbed the membrane is positively charged. This view meets a difficulty in the fact that as a general rule membranes are negatively charged when in contact with neutral water. The adsorption hypothesis meets this difficulty with an additional assumption; namely, that in a neutral solution the OH ions have a greater tendency to be adsorbed by a membrane than the H ions. We should, however, be forced to assume that the preferential adsorption of OH ions occurs also in some cases in acid solutions, since we have seen that collodion membranes (not treated with a protein) are negatively charged even in strong acid solutions.[3] Another difficulty was pointed out by Perrin himself, namely that no other monovalent ion except the H and OH ions were able to reverse the sign of charge of a membrane, and he intimated that this might be due to the fact that the velocities of H and OH ions are greater than those of any other ion. But if the velocity determines the relative degree of adsorption of ions then the H ions should be more readily adsorbed by a membrane in neutral solutions than the OH ions.

A second view, which is held chiefly by physicists, considers the formation of an electrical double layer at the boundary of membrane and water as a case of contact electricity, which may be influenced but which need not be caused by ions. Lenard[4] has shown that when very minute particles are torn off from the free surface of water the minute particles are negatively charged while the water assumes a positive charge. He concludes from this that at the surface of the water there exists an electrical double layer the external stratum of which is negatively charged, while the internal stratum is positively charged. Since he was able to show that such a double layer exists at the surface of water even in a vacuum, this double layer cannot

[3] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 255.

[4] Lenard, P., *Ann. Physik*, 1915, xlvii, 463.

have its origin in an adsorption of ions. He assumes that the double layer at the boundary of water and membrane is also entirely inside the watery phase. This seems, however, to be disproved by an experiment of Coehn and Franken.[5] A sphere of paraffin dipped into water possesses a high negative charge which can be demonstrated electrometrically when the paraffin is lifted out of the water. If, however, a film of water is caused to adhere temporarily to the surface of the paraffin when it is lifted out of the water, the electrometer indicates no charge as long as this film exists, but indicates a charge the moment the film has disappeared. This leaves no doubt that when a solid is bounded by water the one charge is in the membrane and the opposite charge in the watery phase. The fact that paraffin and solid substances in general are negatively charged when in contact with water is ascribed by Coehn to the difference in the dielectric constant of the two phases. Coehn has found that substances of a higher dielectric constant assume a positive charge when in contact with a substance of a lower dielectric constant. This would explain why membranes in general assume a negative charge when in contact with water since the dielectric constant of the latter is relatively high.

The formation of a double layer at the boundary of two phases is thus, according to Coehn, a phenomenon of contact (or frictional) electricity. Lenard and more recently Frenkel[6] have offered suggestions concerning the origin of the double layer which make it dependent on the Rutherford model of the atom. Lenard points out that the atoms at the surface of a body are generally oriented in such a way that the electrons are at the surface and the more massive part (the positive nucleus) is towards the interior. This idea has been elaborated by Frenkel[6] into a theory of surface electric double layers of solid and liquid bodies. According to this theory "double layers must exist on the surface of all liquid and amorphous solid bodies, whatever their chemical constitution. The latter will determine but the magnitude and distribution of electric charges on both sides of the surface."

[5] Coehn, A., and Franken, J., *Ann. Physik*, 1915–16, xlviii, 1005.
[6] Frenkel, J., *Phil. Mag.*, 1917, xxxiii, 297.

On the basis of this theory it seems natural that membranes should as a rule assume a negative charge when in contact with watei. The fact which requires a further explanation is the possibility of a reversal of this sign of charge.

Perrin has shown that in certain cases acids are able to cause a membrane in contact with water to be positively charged. The fact that addition of alkali to an acid solution restores the original negative charge to such a membrane was explained by Perrin on the assumption that in an alkaline solution the OH ions are adsorbed. This latter assumption seems unnecessary since the addition of alkali to an acid may merely serve to lower the hydrogen ion concentration below the level required to make the membrane positive. It is, therefore, only necessary to explain why hydrogen ions in sufficient concentration impress a positive charge on so many membranes and the following is a contribution to the solution of this problem.

We have seen in the preceding papers of this series that a collodion membrane not treated with a protein always assumes a negative charge when in contact with a watery solution, even if this solution is acid. This was demonstrated in two ways, first by electrical endosmose,[1] and, second, by common osmosis.[2] The experiments with electrical endosmose do not lend themselves so well to the exact determination of that hydrogen ion concentration at which the membrane becomes positive on account of electrolysis and the secondary chemical reactions at the electrodes. The method of common osmosis is free from this source of error. When we separate a watery solution from pure water by a collodion membrane the pure water diffuses into the solution at a rate which is not only a function of the gas pressure of the solute but also of the electrostatic forces of the ions in solution. The rate increases with increasing valency of the anion and diminishes with increasing valency of the cation. When, however, the collodion membrane has been treated for some time with a protein it assumes a positive charge when the hydrogen ion concentration exceeds a certain value; and in this case the watery phase of the double layer assumes a negative charge. This is proved by the fact that when we separate a watery solution with a sufficiently high hydrogen ion concentration from pure water by a collodion membrane

treated with a protein, the water will diffuse into the solution at a rate increasing with increasing valency of the cation and diminishing with increasing valency of the anion. It is not difficult to find out by this method the critical hydrogen ion concentration at which the reversal in the sign of charge of the membrane occurs. The experiments to be described in this paper have led to the result that this critical hydrogen ion concentration is connected but not identical with the isoelectric point of the protein with which the collodion membrane has been treated.

Proteins are amphoteric electrolytes which can form salts with metals (metal proteinates, *e.g.* Na proteinate) as well as with the anions of acids (protein-acid salts, *e.g.* protein chloride). Whether they do the one or the other depends upon the hydrogen ion concentration of the solution. Below a certain hydrogen ion concentration the proteins form salts of the type of metal proteinates, *e.g.* Na gelatinate; above this critical hydrogen ion concentration they form salts of the type of protein-acid salts; *e.g.*, gelatin chloride.[7] Between the two concentrations proteins form salts with neither cation nor anion, and this is the so called isoelectric point. For a number of proteins (gelatin included) the isoelectric point lies in the neighborhood of a hydrogen ion concentration of 2×10^{-5} N; for oxyhemoglobin it lies at a very different hydrogen ion concentration; namely, 1.8×10^{-7} N. We will show in this paper that the hydrogen ion concentration required to produce a positive charge in a collodion membrane previously treated with a protein varies in the same sense as the isoelectric point of the protein used.

II. Membranes Treated with Gelatin.

The isoelectric point of gelatin is, according to Michaelis, at a hydrogen ion concentration of about 2×10^{-5} N (or in Sörensen's logarithmic symbol, pH = 4.7). A 1 per cent solution of gelatin was put into a collodion bag over night and was removed the next morning. The bag was rinsed a number of times (ten or twenty times or more) with water to remove all the gelatin except that film which remained apparently attached to the inner side of the col-

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[7] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 39, 237, 363, 483, 559.

lodion membrane. When such membranes were used for osmotic experiments it could be shown that the membrane had a positive charge as soon as the hydrogen ion concentration exceeded 10^{-4} N or a value slightly below 10^{-4}. The point of reversal was therefore near the isoelectric point of gelatin but on the acid side of that point.

The reader will remember that it had been shown in the preceding papers that when we separate a solution of an electrolyte by a collodion membrane from distilled water the water will be attracted by that ion which has the opposite sign of charge and will be repelled by the ion which has the same sign of charge as the watery phase of the double layer, and that both the attractive and repulsive effects increase generally with the valency of the ion.[1] In the case of a salt like $CaCl_2$ the repulsive effect of Ca upon positively charged water prevails over the attractive effect of Cl upon such water and no diffusion of such water into the $CaCl_2$ solution will occur; while when the water is negatively charged the attractive effect of Ca prevails over the repulsive effect of the Cl ion upon the water and water will diffuse rapidly into the solution. We therefore can use a solution of $CaCl_2$ to find out at which hydrogen ion concentration the reversal of sign in the charge of the watery phase occurs. When we separate a $M/256$ solution of $CaCl_2$ from a solution of water by a collodion membrane, water will commence to diffuse into the solution as soon as the water in contact with the membrane assumes a negative charge; while otherwise practically no such diffusion will occur. In these experiments the collodion flasks described in the previous experiments were used. They were closed with rubber stoppers perforated by a glass tube with a bore of 2 mm. in diameter serving as a manometer. The collodion bags were dipped into beakers filled with water. The $M/256$ solution of $CaCl_2$ and the water in the beaker into which the bags were dipped were always given the same hydrogen ion concentration; they were rendered acid by the addition of HNO_3 and alkaline by the addition of KOH. We can in this way ascertain at which hydrogen ion concentration the water will commence to diffuse into the solution of $M/256$ $CaCl_2$ and this will give us that hydrogen ion concentration where the reversal of the sign of charge on the membrane occurs. Such a curve is given in Fig. 1. The abscissæ are the hydrogen ion concentrations (expressed in terms of pH), the ordinates the rise of

level of liquid in the glass tube after 20 minutes. The reader will notice that the curve for M/256 $CaCl_2$ is low and flat as long as the pH > 4.0 and that a sharp rise in the curve begins at a pH of about 4.0 or a little less. The curve rises steeply with diminishing pH (*i.e.* increasing hydrogen ion concentration) until it reaches a maximum at

Collodion membrane treated with gelatin
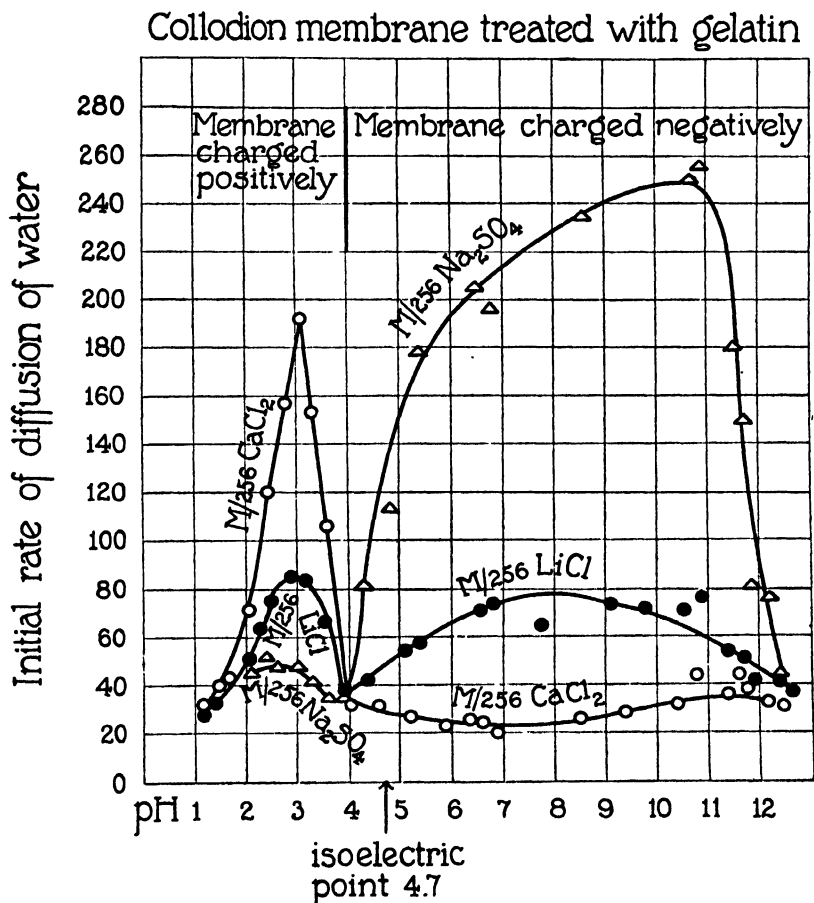
FIG. 1. *Collodion membranes previously treated with 1 per cent gelatin solution.* Abscissæ, pH (logarithms of hydrogen ion concentration with minus sign omitted). Ordinates, initial rate of diffusion of water from pure water through collodion membranes into solutions of salt indicated. Reversal of sign of charge of membrane at pH = 4.0; *i.e.*, on the acid side of the isoelectric point of gelatin.

about pH = 3.1 and then drops again. This drop is the concentration effect discussed in preceding papers and due to the ions with the opposite sign of charge as that of the membrane, in this case Cl and NO$_3$ (the latter being added with the nitric acid).[1]

Instead of using CaCl$_2$ we can also use a M/256 solution of Na$_2$SO$_4$ as a test for the sign of the electrification of water. We put M/256 Na$_2$SO$_4$ into the collodion flask and dip the latter into H$_2$O. The outside water as well as the M/256 solution of Na$_2$SO$_4$ is brought to the same pH by adding HNO$_3$ (or KOH). The SO$_4$ ion attracts positively charged water and the Na ion repels it, but the attractive effect of the SO$_4$ ion is greater than the repelling effect of the Na ion. Water will commence to be attracted by M/256 Na$_2$SO$_4$ as soon as the water is positively charged; when water is negatively charged it will be repelled more powerfully by SO$_4$ than it will be attracted by Na. Hence the hydrogen ion concentration at which the water commences to be positively charged will be indicated by a rise in the level of liquid in the glass tube serving as a manometer. Fig. 1 shows that water commences to be positively charged at a pH of about 4.0 or slightly above where the turning point was also found when we used M/256 CaCl$_2$ solution as a test.

We can finally use solutions with monovalent anion and monovalent cation as a test solution; e.g., M/256 LiCl. In this case we get an attraction for water both in alkaline and in acid solutions. In the acid solution the negatively charged water is attracted by the Li ion and in the alkaline solution the positively charged water is attracted by the Cl ion. Between the two effects there should be a point where the water is neither positive nor negative and hence is not attracted by either ion. Fig. 1 shows that this point lies again at a pH near 4.0.

Fig. 2 gives the point of reversal in the presence of M/256 solutions of NaH$_2$PO$_4$, Na$_2$ oxalate, and NaCl. The point of reversal lies between 4.4 and 4.0, but the steep rise of the curve commences at pH 4.0.

Experiments with a number of other salts and other acids were made, all giving the same result; namely, that the membrane commences to be distinctly positively charged as soon as the pH is 4.0 or slightly above, but below 4.7. At the isoelectric point, pH = 4.7, no acid combines with the gelatin and no change of the sign of charge

can be expected at this point. When the hydrogen ion concentration of the solution becomes higher than this, an increasing amount of gelatin is transformed into gelatin-acid salt. With an increase in the concentration of the gelatin-acid salt an increasing proportion of the surface of the membrane assumes a positive charge. Hence the steep

Collodion membrane treated with gelatin
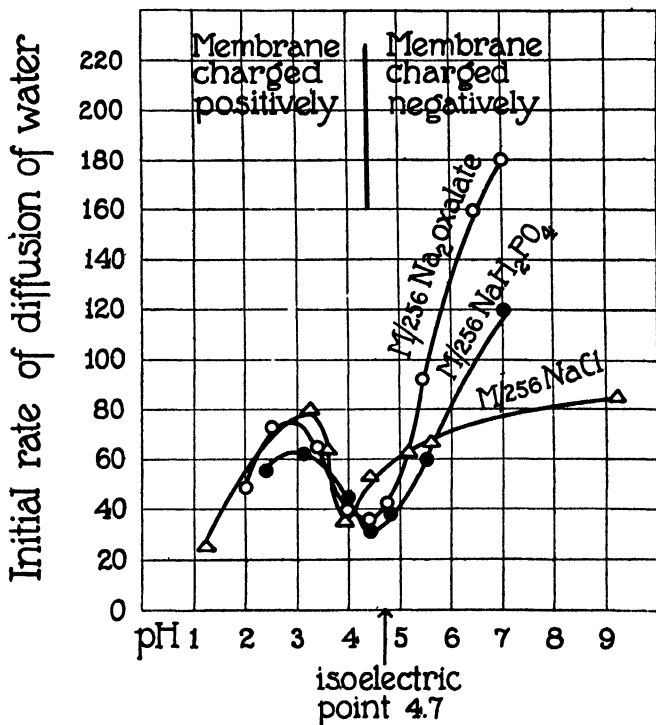
FIG. 2. *Collodion membranes treated with gelatin.* Reversal of sign of charge of membrane on acid side of the isoelectric point of gelatin.

rise of the curves in which $CaCl_2$ or $MgCl_2$ are used as test solutions must lie near the isoelectric point but on the acid side from the latter.

It may be pointed out that the influence of acids on the reversal of the sign of charge is not an additive effect of the oppositely charged

ions, as was the case in regard to the influence of ions on the density of charge of the membrane.[1] If this were the case, SO₄ should shift the steep rise of the curve more to the acid side and Ca more in the opposite direction. The curves given in this paper (and many other curves not published) show that this is not the case. This supports the view that the mechanism for the influence of ions on the density of charge and for the influence of the hydrogen ions on the reversal of the sign of charge is not the same.

The curves in Fig. 1 show that there are three relative minima of the charge of the membrane. One lies at about pH = 4.0 or slightly above, when the membrane is neither positively nor negatively charged. The drop leading to this minimum is not an additive function of the oppositely charged ions and differs in this respect from the drop leading to the other two minima, the latter being due to the diminution of the density of charge on the membrane caused by that ion which has the opposite sign of charge as the membrane. We have shown in the preceding experiments[1] that this depression occurs when the concentration of the electrolyte exceeds a certain value. One of these minima lies on the acid side, namely at a pH less than 2, owing to the fact that the concentration of the HNO₃ added is M/100 or more. This minimum is due to the depressing effect of the anions (NO₃ and SO₄) of the solution upon the density of the positive charge of the membrane. The third minimum lies at a pH of about 12 or above and is caused by the depressing effect of the cation of the solution, Na, K, etc., upon the density of the negative charge of the membrane. If we had continued to increase the concentration of acid there would have been another rise of the curve due to the gas pressure effect of the acid solution; and the same might have happened if we had been able to increase the concentrations of the KOH added; strong alkali solutions, however, dissolve the membrane.

III. Membranes Treated with Casein and Egg Albumin.

The isoelectric point of casein is, according to Michaelis,[8] identical with that for gelatin, since it lies at a pH of about 4.7. When we treat collodion membranes over night with a 1 per cent casein solution in-

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[8] Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 1914.

stead of with gelatin solution, and make the same experiments as described for gelatin, we should expect to find the hydrogen ion concentration at which the collodion membrane treated with casein is rendered positive to be identical with that found for membranes treated
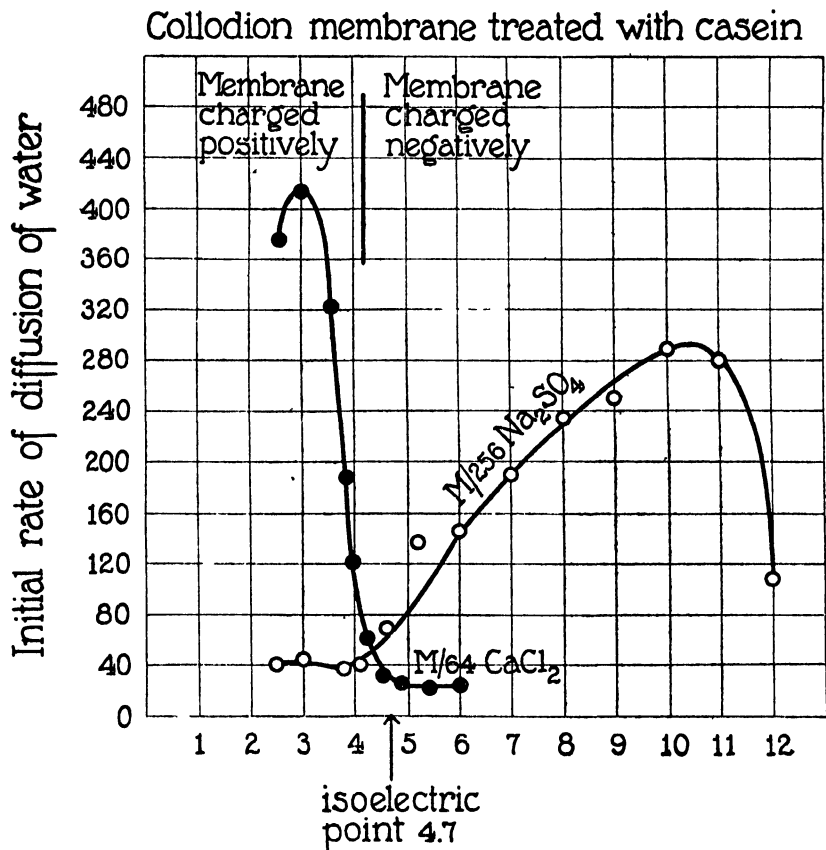
FIG. 3. *Collodion membranes treated with casein.* Reversal of sign of charge of membrane at pH below 4.7, on the acid side of the isoelectric point of casein.

with gelatin. Fig. 3 shows that this is correct. The curves for membranes treated with casein show a steep rise at a pH of 4.0 or slightly above, but below 4.7. The same is true for membranes treated with egg albumin (Fig. 4). The curves show a steep rise
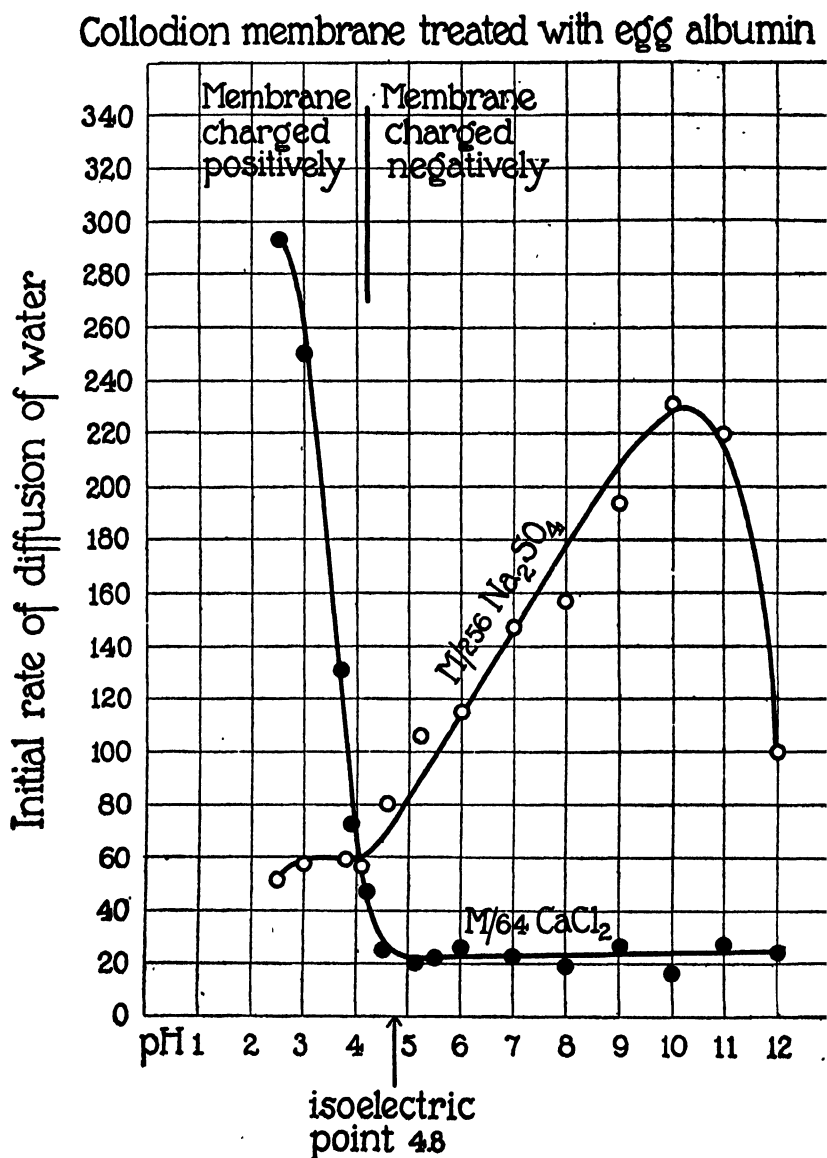
FIG. 4. *Collodion membranes treated with egg albumin.* Reversal of sign of charge of membrane at pH near 4.0, on the acid side of the isoelectric point of egg albumin.

at a pH of about 4.0.   The isoelectric point of egg albumin is, according to Sörensen,[9] at 1.5×10^{-5} N or pH about 4.8.

IV. *Membranes Treated with Oxyhemoglobin.*

Defibrinated ox blood was diluted repeatedly with isotonic solutions of NaCl and centrifuged to remove practically all the proteins
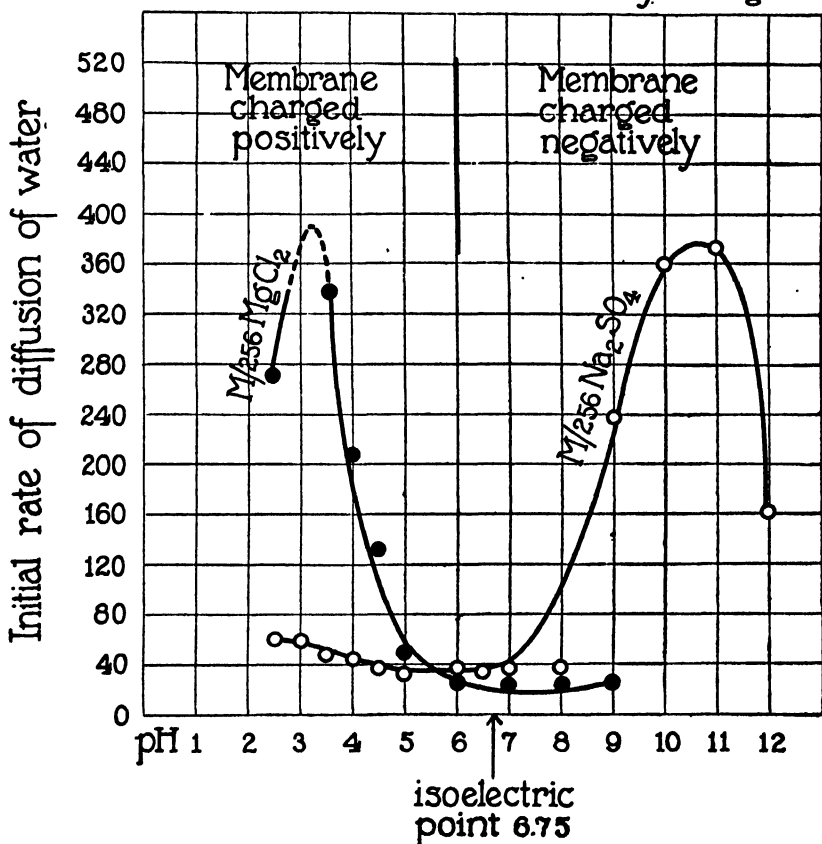
Fig. 5. *Collodion membranes treated with oxyhemoglobin.*   Reversal of sign of charge of membrane at pH of about 6.0, on the acid side of the isoelectric point of oxyhemoglobin.

[9] Sörensen, S. P. L., *Compt. rend. trav. Lab. Carlsberg*, 1915–17, xii.

of the blood except those contained in the red corpuscles. After repeating this process five or more times the corpuscles were laked by adding four times their volume of distilled water. Newly prepared
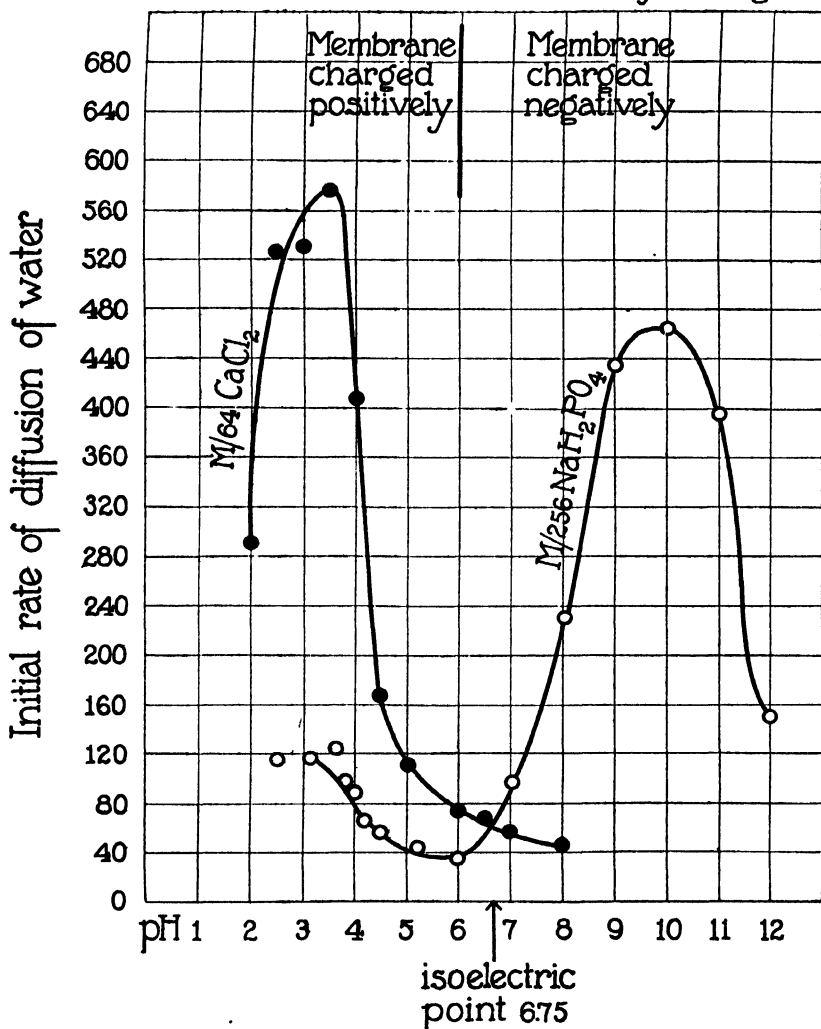
FIG. 6. *Collodion membranes treated with oxyhemoglobin.* Reversal of sign of charge at pH of about 6.0, on the acid side of the isoelectric point of oxyhemoglobin.

collodion bags were filled over night with this crude oxyhemoglobin solution and the bags were used the next day for similar experiments to those described for membranes treated with gelatin. Fig. 5 shows the results of such experiments. The $MgCl_2$ curve begins to rise at a pH of 6.0 while the curve for Na_2SO_4 rises at a pH of about 7.0 or 8.0. Hence when the membrane has been treated with oxyhemoglobin a much lower hydrogen ion concentration is required to induce a positive charge on the membrane than when the membrane has been treated with gelatin. This was to be expected if the reversal of the sign of charge of a membrane treated with a protein depends on the isoelectric point of the protein. According to Michaelis the isoelectric point of oxyhemoglobin is pH = 6.7 to 6.8, and we notice accordingly that the membrane treated with oxyhemoglobin assumes a positive charge when the pH is a little less than 6.7; e.g., 6.0. In Fig. 6 the curve for NaH_2PO_4 shows a distinct rise at a pH of 6.0 and that for $CaCl_2$ at about 6.0 or a little less. In judging the curves in Figs. 5 and 6 the reader must bear in mind that the CO_2 of the air lowers the pH during the experiments and although the experiments lasted only 20 minutes the error so caused was noticeable in the neighborhood of the point of neutrality. Thus the beginning of the rise in the curve for Na_2SO_4 was in reality not at pH = 8, but at a lower pH, either 7.0 or probably less.

V. Collodion Membranes not Treated with a Protein.

When we use membranes *not* treated with gelatin and repeat the experiments described in Fig. 1, we get altogether different results (Fig. 7). There is no minimum or drop at pH of about 4.0 or 6.0 since the sign of charge of collodion membranes not treated with proteins is not reversed by acid.[3] Collodion membranes not treated with a protein are always negatively (and the watery phase positively) charged within the range of our experiments. Hence a $M/256$ solution of $CaCl_2$ cannot attract pure water through such a membrane even in acid solution and the curve for the effect of $M/256$ $CaCl_2$ is flat for the whole range of hydrogen ion concentrations (up to 10^{-2} N). The curve for $M/256$ Na_2SO_4 does not reach a minimum at pH 4.0 but drops slowly reaching a minimum at pH 2.0 or below, this drop being due to the depressing effect of the high concentration of the cat-

ions Na and H of the solution on the negative charge of the membrane. The drop of the curve at the alkaline end (pH = 10 to 12) is due to the high concentration of the cations Na and K, the latter introduced with the KOH added.
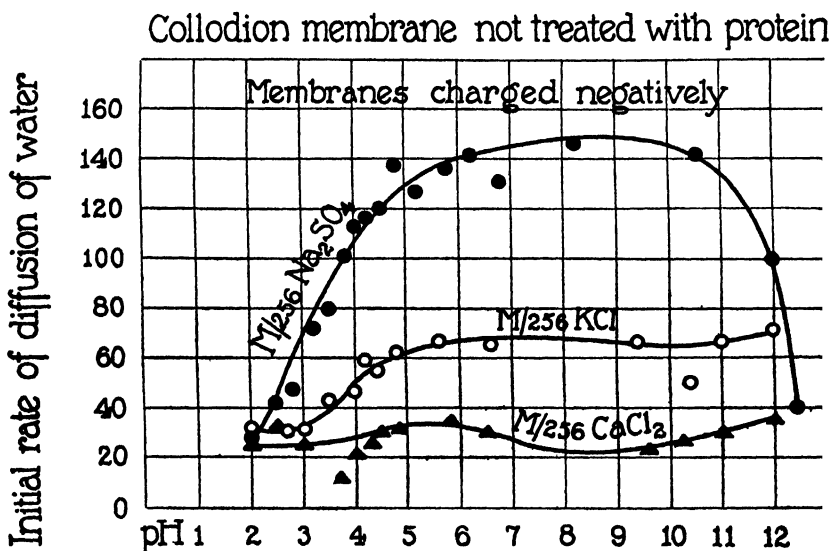
FIG. 7. *Collodion membranes not treated with protein.* No reversal of sign of charge of membrane.

VI. Theoretical Remarks.

Collodion membranes treated with oxyhemoglobin in the way mentioned remain red after they are washed, showing that the treatment of the membrane with a protein solution results in the formation of a durable protein film at the surface of the collodion membrane and probably also in the interstices of the membrane, since traces of oxyhemoglobin or at least of red pigment diffuse out of the membrane. Applying Werner's idea concerning the combination between acid and NH_3 to proteins we should expect that acids are added to an amino-acid group of a protein in a way similar to that in which they are added to NH_3; namely (if the acid added is HNO_3), by forming a salt of the type $R - N \big\langle {}^{HNO_3}_{H_2}$ which dissociates into a positive $R - NH_4$ ion

and a negative NO₃ ion.   On this assumption, the double layer at the boundary of the protein film and a solution would appear to be ionic in character, the positive stratum of the double layer being formed by the protein cations of the surface films of the membrane, while the mobile, watery stratum of the double layer contains the anions of the protein-acid salt.   On this assumption it is clear why the hydrogen ion concentration required to make the membrane positive varies in the same sense as the isoelectric point of the protein used and why it is always higher than that of the isoelectric point, since a certain part of the surface of the protein film must be transformed into protein-acid salt before the membrane assumes a positive charge.   At a hydrogen ion concentration below that of the isoelectric point the protein film is negatively charged since the protein exists here in the form of metal proteinate dissociating into a protein anion and a metal ion, the latter forming the positive watery stratum of the double layer, while the membrane owes its negative charge to the protein anion.

It does not follow, however, that this transformation of protein into protein-acid salt is the only, or even the essential, condition for the reversal of the sign of charge of the double layer by acid. Since acid can bring about a reversal in the electrical double layer of water bounded by air (or possibly even in a vacuum), it is obvious that the modification of the surface layer of the water which is in contact with the membrane may also play a rôle in the reversal of the sign of charge of the protein film.   This possibility has to be considered also in view of the fact that trivalent or tetravalent cations can bring about a reversal in the sign of charge of a membrane treated with protein, even when the hydrogen ion concentration excludes a salt formation between protein and trivalent cation. These facts will be discussed in a subsequent paper.

SUMMARY.

1. It had been shown in previous papers that when a collodion membrane has been treated with a protein·the membrane assumes a positive charge when the hydrogen ion concentration of the solution

with which it is in contact exceeds a certain limit. It is pointed out in this paper that by treating the collodion membrane with a protein (*e.g.* oxyhemoglobin) a thin film of protein adheres to the membrane and that the positive charge of the membrane must therefore be localized in this protein film.

2. It is further shown in this paper that the hydrogen ion concentration, at which the reversal in the sign of the charge of a collodion membrane treated with a protein occurs, varies in the same sense as the isoelectric point of the protein, with which the membrane has been treated, and is always slightly higher than that of the isoelectric point of the protein used.

3. The critical hydrogen ion concentration required for the reversal seems to be, therefore, that concentration where enough of the protein lining of the membrane is converted into a protein-acid salt (*e.g.* gelatin nitrate) capable of ionizing into a positive protein ion (*e.g.* gelatin) and the anion of the acid used (*e.g.* NO_3).

THE INFLUENCE OF THE SUBSTRATE CONCENTRATION ON THE RATE OF HYDROLYSIS OF PROTEINS BY PEPSIN.

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In contrast to the numerous papers on the influence of changes in the pepsin concentration, the influence of varying the protein concentration on the rate of digestion of protein has been but little studied. Weis[1] found that the rate was nearly directly proportional to the protein concentration in low concentration but increased more slowly than the latter in concentrations of more than 2 to 3 per cent. The experiments were made with a crude enzyme preparation which contained several proteolytic enzymes, and were made in such a way as to compare the changes in different solutions after the same time interval, instead of comparing the times required to cause an equal change. They are therefore difficult to interpret.

Preliminary experiments made with a purified pepsin and purified egg albumin showed in general the same results as those found by Weis. In concentrations of more than 2 to 3 per cent the rate of digestion increases more slowly than the protein concentration and finally becomes nearly independent of it. This phenomenon is a very general one in enzyme reactions and many explanations have been offered to account for it. Brown[2] suggested that the relative decrease in the rate of digestion with increasing substrate concentration was due to the fact that the enzyme remained combined with the substrate for a period of time large compared with the time necessary for combination to take place. The enzyme therefore becomes more and more "saturated" with substrate as the relative concentration of substrate to enzyme increases. Van Slyke and Cullen[3]

[1] Weis, *Med. Carlsberg Lab.*, 1903, v, 127.

[2] Brown, A. J., *J. Chem. Soc.*, 1902, lxxxi, 373.

[3] Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1914, xix, 141.

showed that an equation might be derived on the assumption of the above mechanism, based on the law of mass action. The validity of this derivation has, however, been questioned by Falk.[4] Bayliss[5] has advocated the view that the combination is due to adsorption and cannot be considered a mass action phenomenon. He states, as do Armstrong and Armstrong,[6] that the fact that the rate of hydrolysis in many cases is nearly independent of the total substrate concentration cannot be explained on the law of mass action, and must be due to some saturation effect of the enzyme with the substrate.

The fact is frequently overlooked that purely chemical catalysis in strictly homogeneous solutions also shows apparent divergences from the mass law. This point is, however, discussed by Bredig,[7] Mellor,[8] Lewis,[9] and especially by Falk.[10] In the hydrolysis of cane sugar by acids, for instance, the rate of hydrolysis is not proportional to the total concentration of acid used but to the hydrogen ion concentration. As is well known, the hydrogen ion concentration in heavily "buffered" solutions is almost independent of the total acid concentration in certain ranges so that the hydrolysis of cane sugar by such a solution would show an analogous behavior to enzyme reactions in that the rate of hydrolysis of the sugar would be nearly independent of the total acid concentration. The apparent discrepancy of the mass law is therefore due to the fact that the "active concentration" (on which the mass law is based) is in many cases not identical with the total concentration. There is a further discrepancy in these cases due to the fact that the rate of hydrolysis in certain concentrations is not proportional to the C_H^+ as determined by the conductivity ratio. This is the so called salt effect and is probably

[4] Falk, K. G., *J. Biol. Chem.*, 1916–17, xxviii, 389.

[5] Bayliss, W. M., The nature of enzyme action, Monograph on Biochemistry, London, New York, Bombay, and Calcutta, 3rd edition, 1914.

[6] Armstrong, E. F., and Armstrong, H. F., *Proc. Roy. Soc. London, Series B*, 1913, lxxxvi, 561.

[7] Bredig, G., *Ergebn. Physiol.*, 1te Abt., 1902, i, 134.

[8] Mellor, J. W., Chemical statics and dynamics, London, 1904.

[9] Lewis, W. C. McC., A system of physical chemistry, London, New York, Bombay, Calcutta, and Madras, 1919, i.

[10] Falk, K. G., (in press).

due to increased activity of the hydrogen ions by the salt. In any case, it is not due to any peculiarity of the catalytic reaction since the same discrepancy is found in comparing the hydrogen ion concentration as determined by the conductivity and E.M.F. methods. The apparent discrepancy between the mass action law and the kinetics of acid catalysis as outlined above is analogous to the case in enzyme reactions where the rate is not proportional to the enzyme concentration.

Acid hydrolysis, moreover, also shows the same peculiarity in regard to the sugar concentration, *i.e.* the rate does not increase directly as the sugar concentration, as expressed in grams or molecules per liter. In the case of acid hydrolysis the rate increases more rapidly than the concentration. Arrhenius[11] has suggested that this behavior is due to the fact that the active concentration of sugar is not correctly expressed by the molecular concentration and has shown that very much better results are obtained if the osmotic pressure of the sugar solution is used as a measure of the active concentration. He further assumes that the acid affects the equilibrium between active and inactive sugar molecules and so accounts for the "salt effect." The same mechanism is assumed to account for the effect of temperature, which is much greater than that predicted by the kinetic theory. This hypothesis, of course, fits the facts, but in the absence of independent evidence is really an assumption of the law of mass action rather than a proof of the law. Several authors have proposed explanations for catalytic reactions on the same basis; *i.e.*, that the catalyst changes the concentration of certain molecules and so increases the speed of the reaction. Stieglitz[12] and his coworkers have been able to verify this hypothesis experimentally in the case of the acid hydrolysis of imido esters. This reaction shows the same peculiarities as that found in many enzyme reactions; namely, the rate is not proportional to the total ester concentration. Stieglitz was able to show, however, that the rate was directly proportional to the concentration of ester ions. He considers that the acid causes the formation of imido ester salts and

[11] Arrhenius, S., *Z. physik. Chem.*, 1899, xxviii, 317.

[12] Stieglitz, J., and collaborators, *Am. Chem. J.*, 1908, xxxix, 29, 164, 402, 437, 586, 719.

thereby increases the concentration of active ions. He was able to confirm this by independent measurement of the ion concentration (by means of the conductivities). It follows, as emphasized by Stieglitz, that if the above mechanism is correct so called catalytic reactions are merely limiting cases of ordinary reactions in which the combination of the "catalyst" with the substrate or with the products of hydrolysis is too small to be measured. It seems probable that enzyme reactions are of the same type. There is no doubt, at least, that the enzyme often combines with the products of the reaction and so shifts the equilibrium. Bodenstein and Dietz[13] have shown experimentally that this is true in certain cases. It would seem better, therefore, to consider enzyme reactions as cases of bimolecular reaction in which one of the products dissociates more or less completely with the liberation of active enzyme; if the dissociation is complete the result would be a monomolecular reaction and, if no dissociation whatever takes place, a bimolecular reaction. Most enzyme reactions are apparently intermediate. The specificity of enzyme reaction thus becomes neither more nor less remarkable than the specificity of any other chemical reaction. (The author has had the privilege of discussing the above points with Dr. K. G. Falk who has reached independently similar conclusions.) It was shown in a previous paper[14] that the above conception of enzyme reactions as applied to pepsin gives a quantitative explanation for the kinetics of the reaction and explains the fact that the rate is not always proportional to the total concentration of pepsin. Arrhenius[15] has pointed out that it also gives the explanation of Schütz's rule and the divergence from the monomolecular law.

It is clear from the brief account of catalytic reactions given above that the same apparent divergences from the law of mass action are to be found in these reactions as in enzyme reactions and that the divergences in many cases at least are caused by the fact that the active concentration is not the same as the total concentration of substance. It seems quite probable that the same explanation applies to both. There is no doubt that the saturation theory is sufficient

[13] Bodenstein and Dietz, *Z. Elektrochem.*, 1906, xii, 605.
[14] Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 471.
[15] Arrhenius, S., *Med. Nobelinst.*, 1908, i.

to explain many of the facts but in the absence of direct evidence it can hardly be considered proved.

According to this theory a certain amount of enzyme can act only on a limited amount of substrate; after this quantity is reached any excess of substrate has no effect on the reaction. It is clear that according to this mechanism it is the ratio of the concentration of substrate to that of the enzyme which causes the relative decrease in the rate of digestion of the substrate as the concentration of substrate increases, and not the actual concentration of substrate present in the solution. If the effect, however, is due to the fact that the active concentration of substrate is not directly proportional to the total concentration then the falling off of the rate of reaction with increasing substrate concentration is independent of the ratio of substrate to enzyme and depends only on the actual concentration of substrate.

It occurred to the writer that this question might be tested experimentally by comparing the rate of digestion of different substrate concentrations when hydrolyzed with different enzyme concentrations. Assume, for instance, that the substrate at concentration 10 S is found to hydrolyze five times as rapidly as the substrate at concentration S, when enzyme concentration E is used. According to the monomolecular formula the substrate at concentration 10 S should digest ten times as rapidly as the substrate at concentration S. The saturation hypothesis would explain this divergence by the assumption that the enzyme becomes saturated with substrate at a concentration of the latter of less than 10 S. In concentration 10 S, therefore, much of the substrate takes no part in the reaction and the rate of reaction is less than the expected. It would be predicted further that increasing the substrate concentration from 10 S to 20 S would have relatively less effect on the rate of reaction than increasing the substrate concentration from S to 2 S. This is true. It follows also on the saturation hypothesis that increasing the enzyme concentration from E to 10 E should have a relatively greater effect on the rate of digestion of substrate 10 S than on the rate of digestion of substrate at concentration S; since it was assumed in accounting for the effect of increasing the substrate concentration that the enzyme (at concentration E) was more saturated with substrate at (substrate) concen-

tration 10 S than at (substrate) concentration S. According to the saturation theory, the rate of digestion in concentration 10 S is limited only by the concentration of enzyme while the rate at concentration S is limited both by the concentration of enzyme and by the concentration of substrate; hence changing the enzyme concentration should have a greater effect at substrate concentration 10 S than at substrate concentration S. The experiments show that this prediction is not fulfilled. The relative increase in the rate of digestion of substrate at concentration S, caused by increasing the concentration of enzyme from E to 10 E, is identical with the relative increase in rate of digestion of the substrate at concentration 10 S, caused by the same increase in enzyme concentration.

If, on the other hand, the relative decrease in rate with increase in concentration of substrate is due to an equilibrium in the substrate solution which causes the concentration of active molecules to differ from the total concentration, the rate of hydrolysis of the substrate at concentration 10 S should be always five times the rate of digestion at concentration S (in the example just discussed), irrespective of the enzyme concentration. Experiments show that this is actually the case. It is necessary, of course, in making such experiments to be sure that the range covered is such that the enzyme cannot be considered saturated in both substrate concentrations. That is, the range of substrate concentrations must be such as to show nearly direct proportionality between the rate of digestion and the substrate concentration in the lower, but not in the higher concentrations of substrate. It is also necessary to measure the time required to cause a constant change in the substrate and not a constant percentage change or the change made in a given time. The failure to recognize this has led to much confusion in discussion of the kinetics of enzyme reactions (*cf*. Bredig).[7]

This is due to the fact that in most enzyme reactions the products retard the action of the enzyme. It will be clear, therefore (irrespective of the mechanism by which this retardation takes place), that comparative results can be obtained only when a constant amount of products is formed. The actual amount of products formed for example by 10 per cent hydrolysis of varying substrate concentrations will be very different. The larger the concentration of substrate the greater the amount of products formed by 10 per cent hydrolysis and the greater

the consequent slowing up of the enzyme due to inhibiting effect of the products. It is also clear that the retardation will be proportionally greater if a small amount of enzyme is present than if a large amount is present (irrespective of the mechanism by which the retardation is affected). The same reasoning holds for the case when the amount of products formed in a given time is taken as the measure of the rate of reaction. This question was discussed fully in a previous paper.[14]

In all the experiments given in this paper, therefore, the rate of digestion is measured as the reciprocal of the time necessary to cause a small absolute change in the substrate concentration. According to the law of mass action as applied to monomolecular reactions the time necessary to cause this change should be nearly inversely proportional to the substrate concentration, provided the change is small compared to the total change in the lowest concentration. If wider variations than this are used it is necessary to calculate the predicted time according to the monomolecular formula. It may appear that the above method of testing the reaction is a very indirect one and that a simpler and more exact method would be to express the course of a single reaction, according to the mechanism proposed, in a single equation. This equation could then be tested experimentally. Such a procedure, however, leads inevitably to an equation with two or more constants, the value of which must be determined from the experiments themselves, so that but little weight can be attached to the agreement of such an equation with the experimental facts. It seems better, therefore, to limit the experimental conditions in such a way as to leave but one variable.

In all the experiments reported in this paper, the changes are within the above limits and the time required to cause a constant change should, therefore (according to the mass law), be nearly inversely proportional to the substrate concentration at the beginning of the reaction. As will be seen this is not the case if the total concentration of protein is considered as the active concentration but is approximately true if the concentration of ionized protein is considered as the reacting mass.

In these experiments the rate of hydrolysis was followed by means of changes in the conductivity of the solution. It is, therefore, necessary to be sure that the production of the same amount of peptone in each of the solutions used causes the same change in conductivity. This was tested experimentally in each experiment by adding 1 cc. of peptone solution (prepared from egg albumin by the action of pepsin) to 25 cc. of the protein solution and determining the change in conductivity. It was found that the addition of an equal amount of peptone to protein solutions of varying concentrations (from 20 to 1 per cent) does cause an equal change in conductivity provided the hydrogen ion concentration of the solution is greater than pH 1.8. If the solution is less acid than this the change in conductivity of the solution on the addition of a constant quantity of peptone in the presence of a large amount of protein is less than that caused in the presence of a small amount of protein. This is obviously due to the buffer action of the protein in high concentration and can be foreseen from the titration curve of the protein.

Fig. 1 gives the results of two experiments on the effect of the pepsin concentration on the relative rate of digestion of protein solutions of different concentration.   In Experiment 1 (Curve I, Fig. 1) 25 cc. of protein solution containing 8, 4, 2, 1, and 0.5 per cent protein, were hydrolyzed at 25° with the addition of (a) 1 cc. of 2 per cent pepsin, and (b) 1 cc. of 0.2 per cent pepsin.   All solutions were brought to a pH of 1.8 with hydrochloric acid.   The time necessary to cause a given change (about 1.4 × 10⁻⁴ reciprocal ohms) in the specific conductivity was determined.[16]   The reciprocal of this
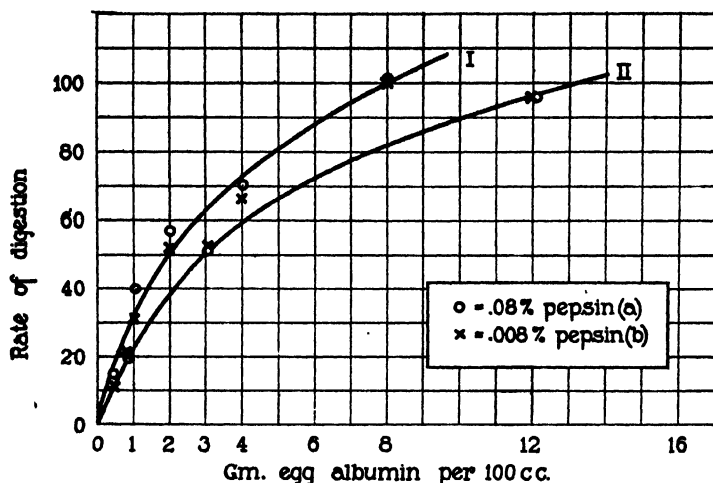
FIG. 1. Relative rate of digestion of egg albumin solutions of different concentration when digested with pepsin solutions of different concentration.

time, therefore, gives the mean rate of digestion of the various solutions for the first 1.4 × 10⁻⁴ reciprocal ohm change.   In order to compare the two series, the rate of digestion of the concentrated egg albumin (in each series) was considered as 100 and the rate of digestion of the other concentrations calculated on this basis.   The curve shows that the relative rate of digestion of the 8 per cent egg albumin compared to the rate of digestion of 4, 2, 1, or 0.5 per cent egg albumin is the same irrespective of whether 2 or 0.2 per cent pepsin was used.   The curve also shows that in low concentrations, 0.5 to 2 per cent, the increase in rate is nearly proportional to the increase

[16] Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 113.

in substrate concentration but increases much more slowly in high concentrations. Experiment 2 (Curve II) shows the same result. It was made the same way but at a pH of 1.6. The points in this case are the average of two determinations. They are therefore more reliable and as the figure shows also more nearly equal. In both experiments the relative activities of the two pepsin solutions were as 4.7:1. It follows from the experiments that this ratio is also inde-

TABLE I.

Influence of Pepsin Concentration on Relative Rate of Digestion of Protein Solutions.

Ratio: $\dfrac{\text{Rate of hydrolysis of 15 per cent albumin}}{\text{Rate of hydrolysis of } \ 1 \text{ per cent albumin}}$ with	
0.08 per cent pepsin.	0.008 per cent pepsin.
8.9	9.1
9.4	9.4
10.0	10.0
9.8	9.7
Average........ 9.52	9.54

Ratio: $\dfrac{\text{Rate of hydrolysis with 0.08 \ per cent pepsin}}{\text{Rate of hydrolysis with 0.008 per cent pepsin}}$ in	
15 per cent protein.	1 per cent protein.
5.3	5.2
5.0	5.4
5.0	5.0
4.7	5.6
5.3	5.5
Average........ 5.06	5.34

pendent of the substrate concentration in which the tests were made. (It was shown in a previous paper[14] that the discrepancy in the rate of digestion as compared with the enzyme concentration can be quantitatively explained on the basis of a mass action equilibrium between the pepsin and peptone.) Table I shows a similar experiment in which several duplicate determinations were made at two protein concentrations with two enzyme concentrations. The results are more accurate and also in closer agreement than those shown in Fig. 1.

It seems necessary to conclude from these experiments that the relative decrease in the rate of digestion of protein solutions of increasing concentration is independent (within the limits of error of these experiments) of the enzyme concentration used.

There does not appear to be any direct experimental evidence on the above point in connection with other enzymes. It is frequently stated, however (Nelson and Vosburgh,[17] Van Slyke and Cullen[8]), that the velocity of reaction is directly proportional to the enzyme concentration under all conditions and irrespective of the substrate concentration. If this is true it follows necessarily that the relative rate of digestion of various substrate concentrations, when hydrolyzed with any given enzyme concentration, is independent of the enzyme concentration used.

It appears to the writer that this is contrary to the result predicted by the saturation theory. According to this theory it would be predicted that the falling off in the increase in the rate of digestion as compared to the increase in concentration of a protein solution (above a certain low concentration) is due to the fact that at this concentration the enzyme begins to become saturated with substrate; *i.e.*, the time necessary for the enzyme to combine with the substrate becomes small compared with the time during which it remains combined. If this saturation effect becomes noticeable at a concentration of protein of 2 per cent with 0.08 per cent pepsin it should become noticeable at a lower protein concentration with 0.008 per cent pepsin. The experiment shows this is not the case. If anything, the figures show that the rate of digestion of the substrate falls off more rapidly (as compared to the concentration) with the higher pepsin concentration than with the lower. In Table I, which is more reliable owing to the larger number of determinations there is less than 1 per cent difference.[18]

It seems necessary to conclude therefore that the relative decrease in the rate of digestion as compared with the increase in protein con-

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[17] Nelson, J. M., and Vosburgh, W. C., *J. Am. Chem. Soc.*, 1917, xxxix, 790.

[18] It probably cannot be assumed that, according to the saturation theory, the rate of digestion (caused by increasing the enzyme concentration ten times) should be increased ten times as much in the concentrated as in the dilute substrate concentration. The increase should be large enough to detect, however.

centration is due to some equilibrium in the protein solution itself and is independent of the enzyme concentration.

It is well known that in acid solution protein exists in an ionized condition. The concentration of ionized protein is not directly proportional to the total concentration but will increase more slowly than the total concentration. It is obvious, therefore, that the rate of digestion will be more nearly proportional to the concentration of ionized protein than to the total concentration of protein. The hypothesis, then, that the ionized protein is the form which takes part in the reaction, will allow a nearer approach to the predicted rate of reaction. Pauli[19] has suggested that the enzyme attacks the ionized protein; there seems, however, to be no direct evidence for this view. It can be tested experimentally by comparing the rate of digestion with the degree of ionization of the protein.

The concentration of ionized protein can be determined approximately from the pH and conductivity measurements. If the total conductivity and the hydrogen ion concentration of a solution are accurately known, the conductivity due to the protein-salt ions can be determined by subtracting the conductivity of the free HCl from that of the solution. The validity of this method rests on three conditions: (1) the conductivity of the free HCl in the solution is the same as that of the same concentration of acid in water solution; (2) the C_{Cl^-} is equal to or greater than the C_H^+; and (3) the hydrogen ion concentration as determined by the E.M.F. method must agree with that found by the conductivity method.[20] The first assumption cannot be tested directly but it has been shown by Hardy[21] and by Loeb[22] that the viscosity of the solution has no significant effect on the conductivity since the viscosity may increase till the solution is nearly solid without an appreciable change in the conductivity. This experiment was repeated and confirmed. The second condition can be shown to hold also by direct measurements of the chlorine ion concentration by means of concentration cells as was done by Manabe and Matula.[23] Many measurements of this kind were made and confirmed those of the above mentioned authors; namely, the chlorine ion concentration is always equal to or greater than the hydrogen ion concentration. It was

[19] Pauli, W., *Arch. ges. Physiol.*, 1910, cxxxvi, 483

[20] For the purpose of these experiments it is only necessary that the conductivity and E.M.F. methods should agree. The absolute value for the C_H^+ is immaterial.

[21] Hardy, W. B., *J. Physiol.*, 1905, xxxiii, 251.

[22] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 559.

[23] Manabe, K., and Matula, J. *Biochem. Z.*, 1913, lii, 369.

found, however, that if the conductivity of the H^+ and the total Cl^- were subtracted from that of the solution the remaining values were within the limits of error of the measurements. In other words, the conductivity due to the protein ion itself is very small compared to that due to the excess chlorine ion (by excess Cl^- is meant the difference between the total C_{Cl^-} and the $C_{Cl} - = C_H^+$). Since, however, the protein ion must equal in concentration the excess chlorine ion the value for the conductivity obtained by subtracting the conductivity of the free HCl from that of the solution may be considered as proportional to the amount of ionized protein. (Recent work, by Noyes, Milner, and others, has rendered questionable the exact interpretation to be put upon conductivity ratios; they very probably do not represent the actual ion concentration in all cases.) The third condition may be experimentally fulfilled by standardizing the apparatus used for the C_H^+ determinations against HCl solutions of known conductivity and taking the C_H^+ as that determined by the conductivity ratios. This method was used in the present experiments. The final values for the conductivity due to the protein-salt ions are the difference between two large figures so that the error is very large and becomes larger as the solution becomes more dilute. Below 1 per cent protein solution (at pH 1.7) the value is meaningless as it usually lies within the limit of error.

The egg albumin was crystallized three times and then dialyzed under pressure at the isoelectric point until the specific conductivity was lower than 1×10^{-4} reciprocal ohm. The solutions were then brought to a pH of 1.6 to 1.8 with HCl and then diluted with HCl of exactly the same pH. The solutions varied from 16 to 1 per cent egg albumin. The time necessary to cause a constant small change in the conductivity of the resulting solution by the same amount of pepsin was then determined as described previously.[16] The reciprocal of this time is plotted in the curves as the rate. The conductivity of the solution was measured on an aliquot part of the solution to which the equivalent amount of inactivated pepsin had been added. The C_H^+ was determined by the E.M.F. method on this solution. The value given for the specific conductivity of the protein is obtained by subtracting the specific conductivity of the free HCl from that of the solution. The experimental error of the value is 5 to 10 per cent in the high concentrations and 20 to 30 per cent in the lower. The figures given are the averages of three determinations. All measurements were made at $25° \pm 0.01$.

The conductivity and rate of digestion of the egg albumin was measured in this way. It was found in general that the conductivity of the protein solution was, within the rather large limits of error, directly proportional to the rate of digestion of the solution. In other words, the rate of digestion is that predicted by the mass law if the ionized protein is considered as the reacting form. The results of three such experiments are given graphically in Fig. 2 in which

the rate of digestion is plotted against the conductivity of the protein. This figure shows that the two values are approximately directly proportional.
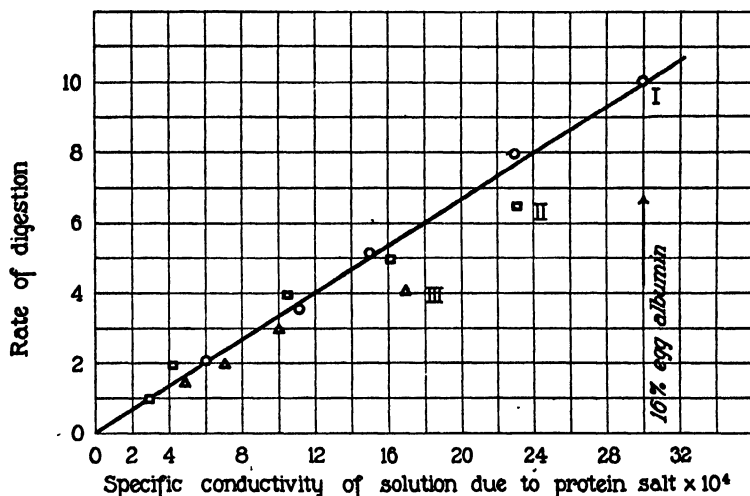
FIG. 2. Rate of digestion and conductivity of egg albumin solutions.

Viscosity of the Solution.

It is impossible to use egg albumin in more concentrated solutions than 16 per cent as the higher concentrations set to a jelly very rapidly. It seemed quite possible that the increasing viscosity of the solution might affect the rate of digestion (as found by Colin and Chaudun[24] for invertase). This question can be tested experimentally by taking advantage of the well known hysteresis of albumin solutions.

500 cc. of a 25 per cent egg albumin solution were titrated to pH 1.6 with HCl placed at 25°, and the viscosity and rate of digestion of a sample determined at intervals for about 10 hours. The amount of pepsin used was such that the viscosity of the digesting solution did not change appreciably during the determination. This was due to the fact that the decrease in viscosity by the pepsin was equalized by the increase of the viscosity with time. The viscosity of the solution at the beginning of the experiment was about three times that of water and at the end too large to measure by the viscosimeter. At the beginning of the last

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[24] Colin, H., and Chaudun, A., *Compt. rend. Acad.*, 1919, clxix, 849.

to the law of mass action between the uncombined pepsin and the protein ion. The mechanism may be formulated as below.

$$\text{Pepsin} + \text{peptone} \leftrightharpoons \text{pepsin-peptone}$$

or

$$C_{\text{pepsin}} = K \frac{C_{\text{pepsin-peptone}}}{C_{\text{peptone}}} \quad (1)[30]$$

and

$$\text{Protein-chloride} \leftrightharpoons \text{protein}^+ + Cl^-$$
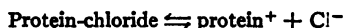
or

$$C_{\text{protein ion}} = K \frac{C_{\text{protein-chloride}}}{C_{Cl^-}} \quad (2)[30]$$

The reaction would be expressed by

$$\text{Protein ion} + \text{pepsin} \Rightarrow [\text{protein ion-pepsin}] \rightleftharpoons \text{peptone-pepsin} \rightleftharpoons \text{pepsin} + \text{peptone}$$

The rate of hydrolysis of the protein at any instant of time would therefore be proportional to the concentration of protein ions and of free pepsin present in the solution at that instant and the differential expression for the rate of reaction would be

$$-\frac{dC_{\text{protein ion}}}{dt} = K\, C_{\text{protein ion}} \cdot C_{\text{pepsin}}$$

where $C_{\text{protein ion}}$ and C_{pepsin} are determined by equations (1) and (2). There is probably little doubt that the enzyme and substrate unite to form an addition product, but according to the experimental evidence found in this paper the time during which they are combined is negligible in the consideration of the kinetics of the reaction.

The mechanism outlined above will explain, at least qualitatively, the peculiarities in the kinetics of other enzyme reactions. It seems very unlikely, however, that the equilibrium in the substrate solution should always be ionic. It may be any isomeric equilibrium. Since,

[30] The equilibrium expressed in (2) is certainly, and that expressed in (1) is probably, influenced by the hydrogen ion concentration.

in such cases, it is extremely difficult to obtain any independent measurement of the equilibrium, there seems to be no way to test the proposed mechanism.

SUMMARY.

1. It is pointed out that the apparent exceptions to the law of mass action found in enzyme reactions may be found in catalytic reactions in strictly homogeneous solutions.

2. These deviations in the rate of reaction from the law of mass action may be explained by the hypothesis that the active mass of the reacting substances is not directly proportional to the total concentration of substance taken.

3. In support of this suggestion it is shown that for any given concentration of pepsin the relative rate of digestion of concentrated and of dilute protein solutions is always the same. If the rate of digestion depended on the saturation of the surface of the enzyme by substrate the relative rate of digestion of concentrated protein solutions should increase more rapidly with the concentration of enzyme than that of dilute solutions. This was found not to be true, even when the enzyme could not be considered saturated in the dilute protein solutions.

4. The rate of digestion and the conductivity of egg albumin solutions of different concentration were found to be approximately proportional at the same pH. This agrees with the hypothesis first expressed by Pauli that the ionized protein is largely or entirely the form which is attacked by the enzyme.

5. The rate of digestion is diminished by a very large increase in the viscosity of the protein solution. This effect is probably a mechanical one due to the retardation of the diffusion of the enzyme.

QUANTITATIVE LAWS IN REGENERATION. II.

BY JACQUES LOEB.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

I.

In a preceding paper[1] it has been shown that, when a piece of stem inhibits the production of roots and shoots in a leaf of *Bryophyllum calycinum* connected with it, the stem gains in mass and this mass equals approximately the mass of shoots and roots the leaf would have produced if it had been detached from the stem. On the basis of this fact it was suggested that the inhibitory influence of the stem upon the formation of roots and shoots in the leaf is due to the fact that the material available for this process naturally flows into the stem.

In these experiments the quantity of roots formed had not been measured directly but had been calculated on the assumption that the dry weight of the roots formed is on the average 42 per cent of the dry weight of the shoots formed in the same leaves. Since this experiment seems to be crucial for the answer to the question why the leaf does not form shoots or roots as long as it forms a part of a normal plant it seemed advisable to make a direct determination of the mass of roots formed by an isolated leaf. Five new sets of experiments, as a rule with eight pairs of sister leaves, were made. The method was the same as that described in the first paper.[1] Table I gives the dry weight of the organs. The experiments lasted about 1 month; if they last too short a time the error in measuring vitiates the result and if we wait too long another complication arises inasmuch as the leaves of the shoots formed become too large and contribute too much material for further growth and regeneration.

[1] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 297.

In Table I, a is the dry weight of roots and shoots formed by the isolated leaves without stems; b is the dry weight of the half stems cut off from these leaves at the beginning of the experiment; a_1 is the dry weight of roots and of shoots formed by the sister leaves left in connection with a piece of half stem. b_1 is the dry weight of the latter half stems at the end of the experiment. According to our theory the inhibitory effect of this piece of stem left in connection with a leaf on the production of roots and shoots by the leaf should be due to the fact that naturally the material (or the greater part of

TABLE I.

Dry Weight of Roots, Shoots, and Half Stems.

Experiment No.	Duration.	a		b	a_1		b_1	$\dfrac{a}{a_1 + b_1 - b}$
		Roots.	Shoots.		Roots.	Shoots.		
	days	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	
1	30	0.169	0.412	0.474	0.039	0.067	1.054	$\dfrac{581}{686} = 0.85$
2	33	0.133	0.408	0.426	0.062	0.158	0.753	$\dfrac{541}{547} = 0.99$
3	33	0.127	0.509	0.415	0.045	0.114	1.034	$\dfrac{636}{778} = 0.82$
4	30	0.127	0.438	0.563	0.053	0.143	1.029	$\dfrac{565}{662} = 0.85$
5	30	0.101	0.416	0.422	0.043	0.162	0.823	$\dfrac{517}{606} = 0.85$

the material) used for root and shoot production in the leaf flows into the stem and is utilized here for growth. We should therefore expect to find that the dry weight of the half stem (b_1) left in connection with the leaf should increase as much as or even more than the difference in the dry weight of the roots and shoots produced in the two sets of sister leaves amounts to. If a is the mass of shoots and roots produced by the leaf isolated completely from the stem, a_1 the mass of roots and shoots produced by the sister leaves left in connection with the half stem, b the mass of the half stems at the beginning of the

experiment, and b_1 the mass at the end, we should expect to find that $b_1 - b \gtreqless a - a_1$ or that $\dfrac{a}{a_1 + b_1 - b} \gtreqless 1$. Table I shows that this is correct. The average ratio of $\dfrac{a}{a_1 + b_1 - b}$ is 0.87.

When a leaf is connected with a stem which prevents regeneration in a leaf the inhibitory effect is therefore due to the fact that the material available for the regenerative growth in the notches of the leaf flows into the stem. In fact 14 per cent more dry weight goes from the leaf into the stem than would go into the growth of the regenerating leaf. For some reason the flow of sap into the regenerating parts in the notches of a leaf seems to be less complete than the flow of available material into the stem.

II.

The material which goes into the stem is used for different purposes; it may cause the growth of an axillary shoot in the stem as in Fig. 1. Part of it causes an increase in the mass of the stem. The stippled lines in Fig. 1 indicate part of the increase in the mass of the stem. This increase is greatest at the basal end where it constitutes the callus.

In a paper previously published[2] the writer has called attention to the fact that when a piece of stem is left in connection with a leaf the inhibitory effect on the formation of roots in the leaf increases with the size of the piece of the stem but less rapidly than this. This is due to the fact that the increase in mass of the stem is not the same throughout the whole length of the stem but that it is greater at the base and possibly also (though to a lesser degree) in the region of a node.

Hence the increase in mass of a stem consists of the sum of two quantities, $c + l\,c_1$, where c expresses the mass of the callus which does not necessarily increase with the mass of the stem, and $l\,c_1$, where l is the length of the stem and c_1 the increase in the mass per cm. length of a stem with unit periphery. It is obvious why the inhibitory power of a stem increases generally with its length but less rapidly.

[2] Loeb, J., *Ann. Inst. Pasteur*, 1918, xxxii, 1.

Figs. 2 and 3 show the distribution of the reddish pigment formed in the leaves suspended in air and drying out., The reddish pigment is indicated by the stippled area in the leaf.   In the two leaves connected with a piece of stem where the regeneration of shoots in the leaf is inhibited the pigment flows into the stem and the newly forming shoots in the stem.   The old leaf contains pigment only in that part which is close to the petiole and this is obviously pigment in
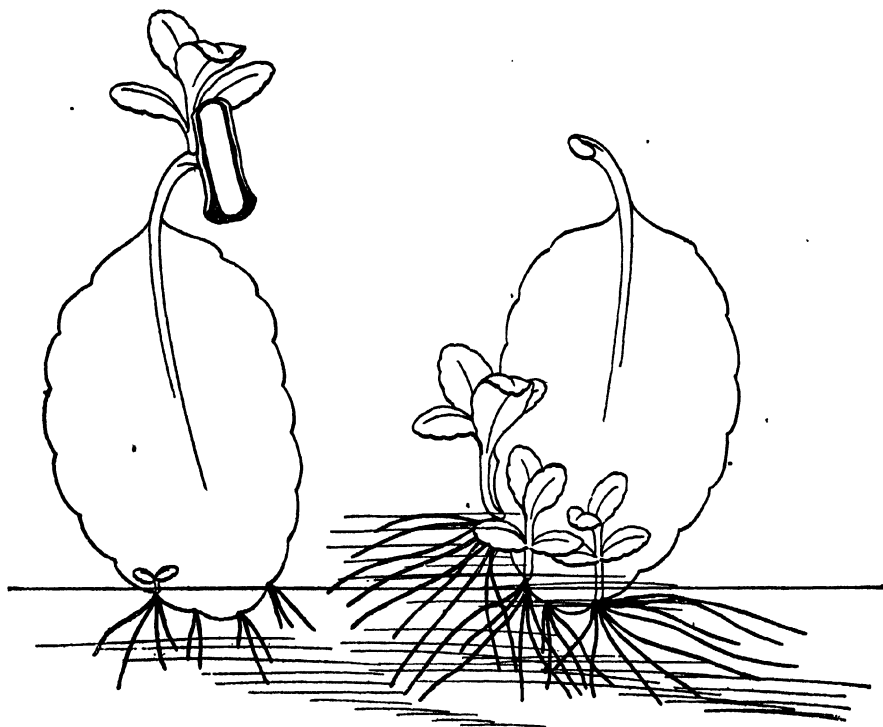
FIG. 1. Leaves suspended with apex in water.   Inhibitory influence of a piece of half stem on shoot and root formation in leaf connected with it.   The leaf to the right (without stem) has formed three vigorous shoots and numerous roots.   The sister leaf with a small piece of split stem attached has formed in the same time a tiny shoot and a few roots.   The material required for root and shoot formation in the leaf has migrated into the stem and gives rise there to an axillary shoot and to the growth in the stem indicated by stippling.   Duration of experiment, Mar. 30 to Apr. 27.

the process of flowing into the stem. The two sister leaves without stems have formed shoots and roots in their notches, one at the base and the other on one side. The reddish pigment is collected near the
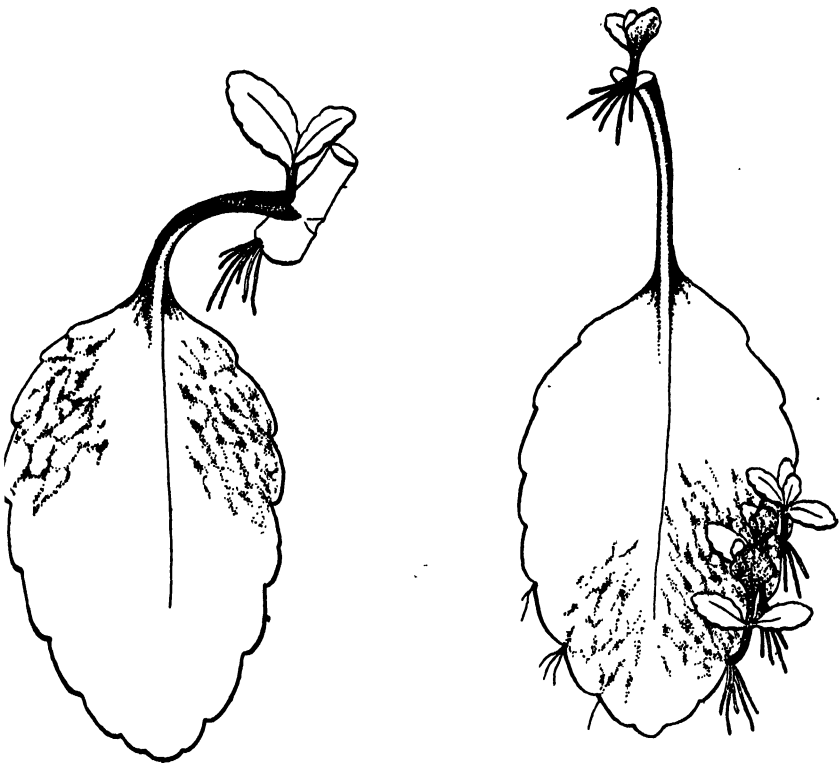
FIG. 2. Two sister leaves suspended entirely in air. The stippling indicates the reddish pigment. In the leaf to the left which is connected with a piece of stem the pigment flows into the axillary shoot of the stem, into the petiole, and is collected in the upper part of the leaf nearest the petiole. In the sister leaf without stem it collects near the place where the new shoots are formed and in the new shoots. Duration of experiment, Feb. 17 to Apr. 5.

newly formed shoots and on its way to these organs. These observations support the assumption that the inhibitory effect of the piece of stem on shoot formation in a leaf is due to the fact that the material available for shoot formation in the leaf flows naturally into the

stem. It also illustrates the principle that when in one part of the leaf growth is very rapid the growth in other parts of the leaf is retarded or inhibited for the reason that the flow of material is towards the rapidly growing organs.

These drawings were made later than those given in a previous paper,[3] which were for this reason less striking.

The inhibitory effect of a small piece of stem on a leaf does not last permanently. It is possible that at first the piece of stem grows in the way described thus inhibiting growth in the leaf, but that later
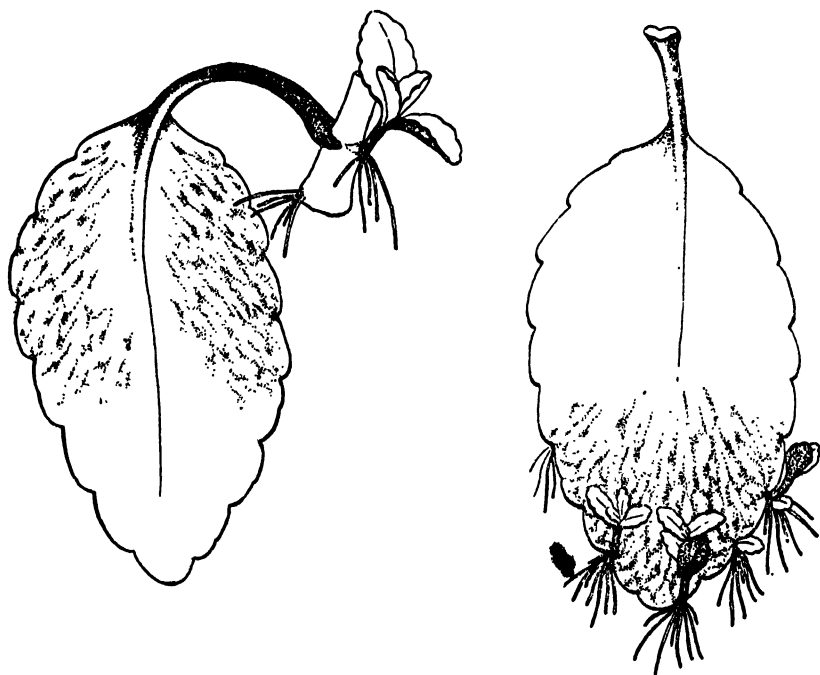
FIG. 3. The same as Fig. 2.

the rate of growth in the piece of stem diminishes, and parallel with this the inhibition of the piece of stem on the growth of roots and shoots in the leaf diminishes also.

[3] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 373.

SUMMARY.

This paper contains the results of a reexamination of a law expressed in a previous paper; namely, that when a piece of stem inhibits the growth of shoots and roots in a leaf connected with it the dry weight of the stem increases and that this gain equals approximately the mass of shoots and roots the leaf would have produced if it had been detached from the stem. This has been confirmed and it has been shown that the gain of the stem as a rule even exceeds slightly the mass of shoots and roots the leaf would have produced if it had not been inhibited by the stem. This supports the idea that the inhibitory influence of the stem upon the formation of roots and shoots in the leaf is due to the fact that the material available and required for this process naturally flows into the stem.

THE REVERSAL OF THE SIGN OF THE CHARGE OF COLLODION MEMBRANES BY TRIVALENT CATIONS.

By JACQUES LOEB.

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I.

The reversal of the sign of charge of collodion membranes (treated previously with a protein) by hydrogen ions has been discussed in a preceding paper[1] and in this paper the influence of trivalent cations on the reversal will be considered. We shall omit the discussion of the action of tetravalent cations (*e.g.* $ThCl_4$) since their solutions have so high a concentration of hydrogen ions that this alone suffices to bring about a reversal in the sign of charge.

We will prove first that collodion membranes which have previously been treated with a protein give the reversal in the sign of charge in the presence of trivalent cations, while collodion membranes not so treated do not show the reversal. When a solution of $CaCl_2$ of not too high a concentration (*e.g.* below $M/8$) is separated from pure water by a collodion membrane, which is negatively electrified, the solution shows no attraction for water, while it attracts water powerfully when the membrane is charged positively. The Ca ion acts as if it repelled positively charged water and as if it attracted negatively charged water. To find out whether or not trivalent cations reverse the sign of charge of the membrane we have to add a low concentration of a "neutral" salt with trivalent cation to the solution of $CaCl_2$. Weak solutions of $CeCl_3$ and $LaCl_3$ satisfy this condition. The solutions of $CaCl_2$ are for this purpose made up in $M/1,024$ solutions of $CeCl_3$ or $LaCl_3$ instead of in distilled water; and the distilled water surrounding the collodion bag is also replaced by

[1] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 577.

M/1,024 CeCl$_3$, so that in regard to CeCl$_3$ or LaCl$_3$ the liquid is the same on both sides of the membrane. Any osmotic effect can therefore only be due to the CaCl$_2$. This allows us to investigate the question of the reversal of the sign of charge of the membrane by the Ce ions. Fig. 1 shows that the CeCl$_3$ can only reverse the sign of charge of the membrane if the latter has previously been treated with gelatin. The lower curve gives the initial rate of diffusion of water (after 20 minutes) into CaCl$_2$ solutions (of different concentra-
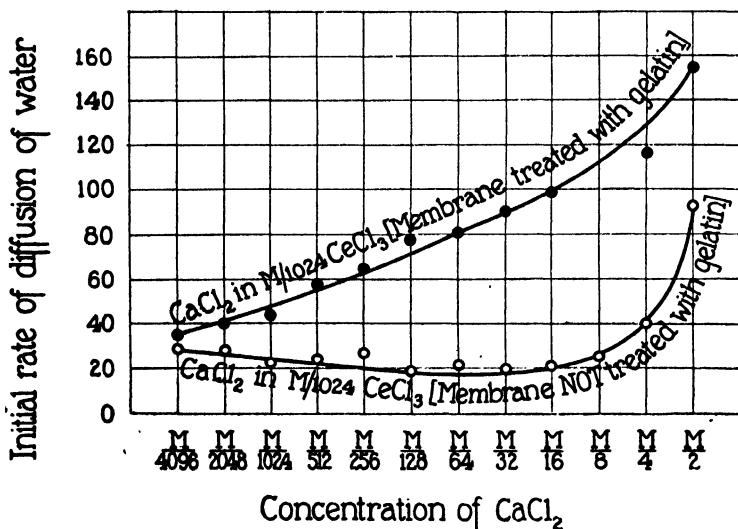
FIG. 1. Showing that CeCl$_3$ renders a collodion membrane positive only when it has been treated with a protein, since CaCl$_2$ below M/16 attracts water only when the membrane is positively and the water negatively charged.

tion) through collodion membranes not treated with gelatin. The membrane is negatively charged since no rise of the level of water in the solution occurs as long as the concentration of CaCl$_2$ is below M/8. The rise of the curve at a higher concentration has a different cause which need not be considered in this connection. The upper curve gives the influence of the same CaCl$_2$ solutions on the rate of diffusion of water from pure water into solution when the membrane has previously received a gelatin treatment. In this case the level of liquid rises in the solution and the more so the higher

the concentration of the CaCl$_2$ solution. The CeCl$_3$ has therefore caused a reversal in the sign of charge of the membrane making the latter positive. This made it possible for the CaCl$_2$ solution to increase the rate of diffusion of water through the membrane into the solution. The pH of the solutions varied between 5.1 (in the lowest concentration of CaCl$_2$) and 5.9 (for the highest concentration); *i.e.*, the solutions were all on the alkaline side of the isoelectric point of gelatin.

Experiments with electrical endosmose confirmed the conclusion that in the presence of CeCl$_3$ the membrane assumes a positive charge when the membrane had previously been treated with gelatin, but that the membrane remains negatively charged when it has not been treated with a protein. In these experiments with electrical endosmose the solutions inside and outside the collodion bag were solutions of CeCl$_3$ of identical concentration.

II.

We have shown in another paper[2] in this number of the *Journal* that in the case of collodion membranes treated with gelatin and rendered positive by acid the rate of diffusion of water from the side of pure water through the membrane to the solution is raised by cations in the order K < Na < Li < divalent cations < trivalent cations. Since the Ce ions render the gelatin film of the collodion membrane positive the influence of different cations on the attraction of water should increase in the same order as when the membrane is rendered positive by acid; and the attraction of water by the solution should be a minimum in the case of K. Fig. 2 shows that this is true. In these experiments different concentrations of the four salts, KCl, NaCl, LiCl, and CaCl$_2$, from M/2,048 to 1M were made in M/1,024 CeCl$_3$. The H$_2$O surrounding the collodion bags containing the solutions was replaced by M/1,024 CeCl$_3$ to eliminate the osmotic effect of CeCl$_3$ in the experiments. The experiments show that the attraction of water by the four salts follows .the order we should expect if Ce caused the membrane to assume a positive charge; *i.e.*, K < Na < Li < Ca. The pH· in these experiments varied from

[2] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 673.

5.2 to 6.0, thereby proving that the Ce reversed the sign of the charge of the gelatin film on the inside of the membrane without raising the hydrogen ion concentration to that point where an acid reversal occurs. In Fig. 3 M/1,024 AlCl₃ solutions are used for rendering the membrane positive. In this case the pH was about 4.1 in all solutions.
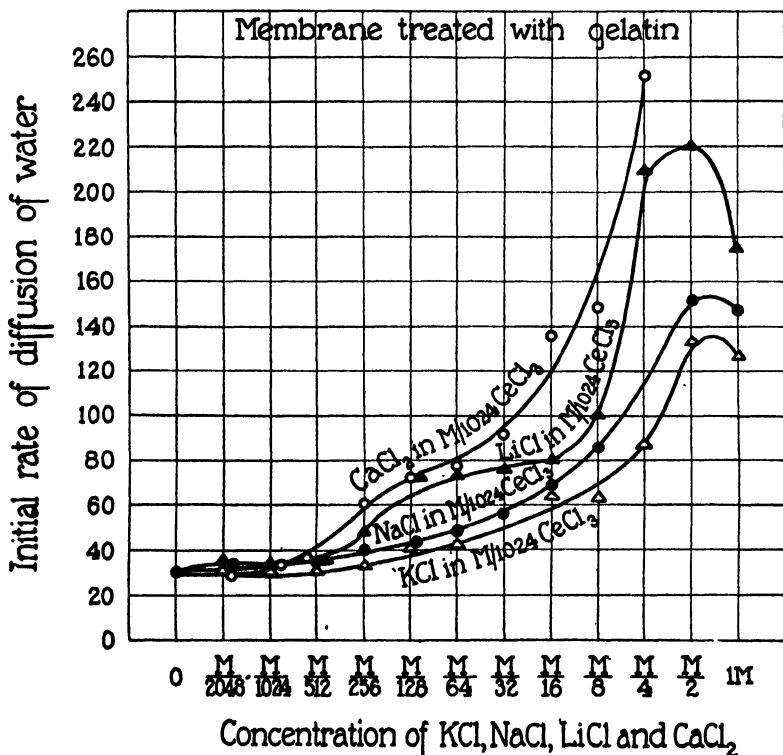
FIG. 2. The relative influence of different cations upon the attraction of water is in the order Ca > Li > Na > K, proving that the membrane is positively charged in the presence of Ce.

The order of efficiency of cations remains the same as when CeCl₃ is used, but the effect is quantitatively larger in Fig. 3 than in Fig. 2.

All these experiments and many others show that trivalent cations cause the membrane previously treated with gelatin to be charged positively on the alkaline side of the isoelectric point of gelatin.

III.

The next question was as to the minimal concentration of a trivalent cation required to render the membrane positive.   For this purpose
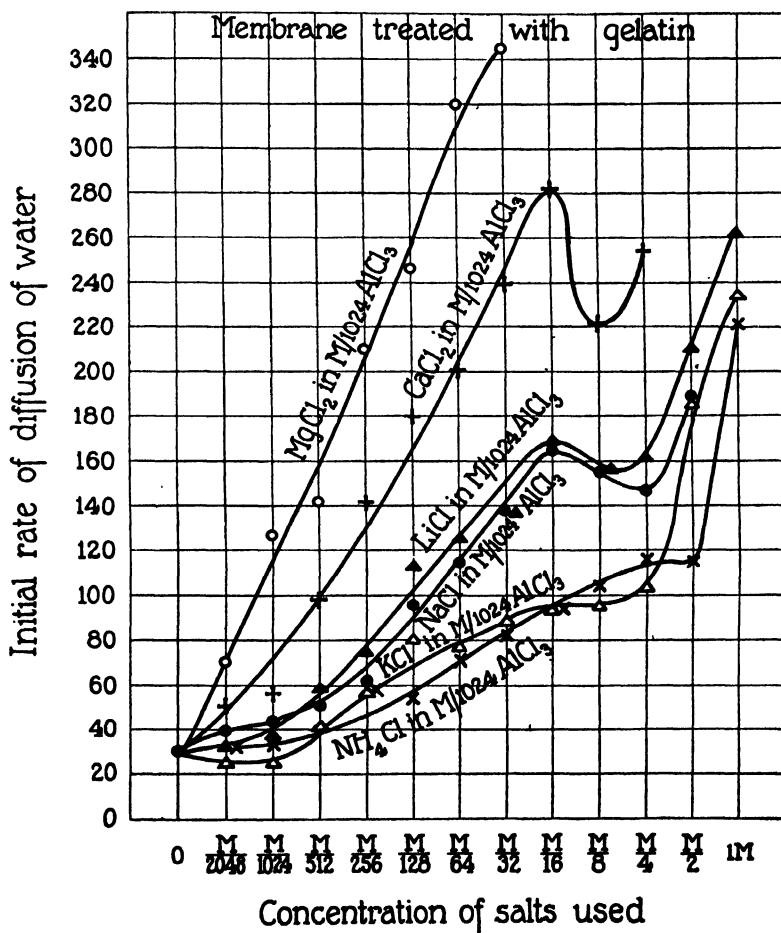
FIG. 3. Showing that Al charges the membrane positively.

various concentrations of CeCl₃ were prepared, beginning with M/65,536 CeCl₃.   To each of these solutions so much CaCl₂ was added that the concentration was M/256 in regard to CaCl₂.   These

solutions were put into collodion bags previously treated with gelatin. The bags were put into beakers containing the same solutions of CeCl₃ as that inside the bag but free from CaCl₂. Hence the Ce acted only on the sign of the charge of the membrane but not on the attraction of water. This latter was done by the CaCl₂. Whenever the level of the solution of CaCl₂ rises we know the membrane must be positively charged. The curve in Fig. 4 shows that the
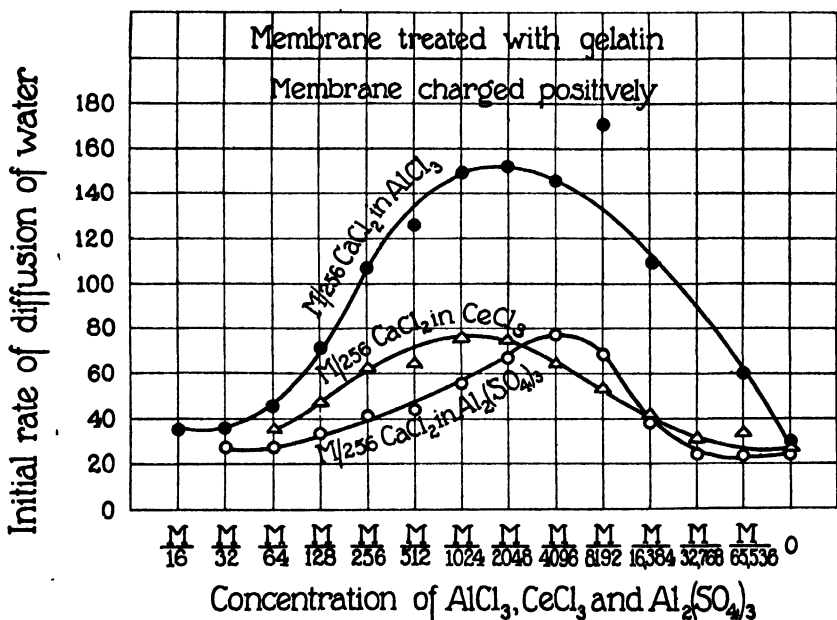
FIG. 4. Lowest concentration at which Ce and Al charge the membrane positively.

membrane already assumes a positive charge in M/32,768 CeCl₃ and probably in lower concentrations of CeCl₃.

When the CeCl₃ was replaced by AlCl₃ the membrane assumed a positive charge when the concentration of AlCl₃ was less than M/65,536. The pH of this latter solution of AlCl₃ was 5.0, so that the reversal was due to the Al ion and not to the hydrogen ion concentration. The fact that Al is more efficient than the Ce (or La) ion may be connected with the fact that the ionic radius of Al is considerably

smaller than that of either Ce or La. The drop in the curves when the concentration of the CeCl₃ or AlCl₃ solution exceeds м/1,024 is due to the concentration of the anion, as is shown by the fact that the drop is greater in the case of Al₂(SO₄)₃ than in the case of AlCl₃. This latter phenomenon has been discussed in previous papers. The pH of the Al₂(SO₄)₃ solutions almost coincided with that of the AlCl₃ solutions of the same concentration.

IV.

It is therefore certain that the trivalent ions bring about a positive electrification in the collodion membrane in contact with water when the membrane has been treated with a protein, but that they cannot reverse the sign of the charge of collodion membranes not so treated. This indicates that the reversal is at least partly due to an action of the trivalent cations on the protein. A chemical combination can only occur between Ce or Al and gelatin on the alkaline side of the isoelectric point where the gelatin is capable of combining with metals. In the experiments described thus far the pH of the CeCl₃ solutions was with one exception always > 4.7. The question arises, how will a CeCl₃ solution act on the acid side of the isoelectric point of the protein forming the inner lining of the collodion bag where the membrane is already rendered positive by the acid?

м/256 LaCl₃ solutions were prepared at different pH, from 7.0 to 2.6, by adding KOH or HNO₃ to the distilled water used for the solutions as required for the pH. These solutions were put into collodion bags and the latter were dipped into H₂O of exactly the same pH as that of the solutions inside the bag. The initial rise of water (in the first 20 minutes) inside the bags was then observed. Similar experiments were made with CeCl₃. The collodion membranes had previously been treated with gelatin. Fig. 5 gives the results. It is plain that the curves consist of two distinct parts and that the dividing line seems to lie near the isoelectric point of gelatin. On the alkaline side of the point, i.e. for pH 4.7 or above, the initial rate of diffusion is near 140 mm.; and it varies very little with a change of pH. As soon, however, as the pH falls to 4.7 or below, the rate of diffusion rises steeply to 300 and 340 mm. for the two salts.

To understand this result we must discriminate between the effects of the trivalent cations on the reversal of the sign of charge and on the increase of the rate of diffusion of water into the solution, which seems to be due to an increase in the density of charge of a membrane already positive. On the alkaline side of the isoelectric
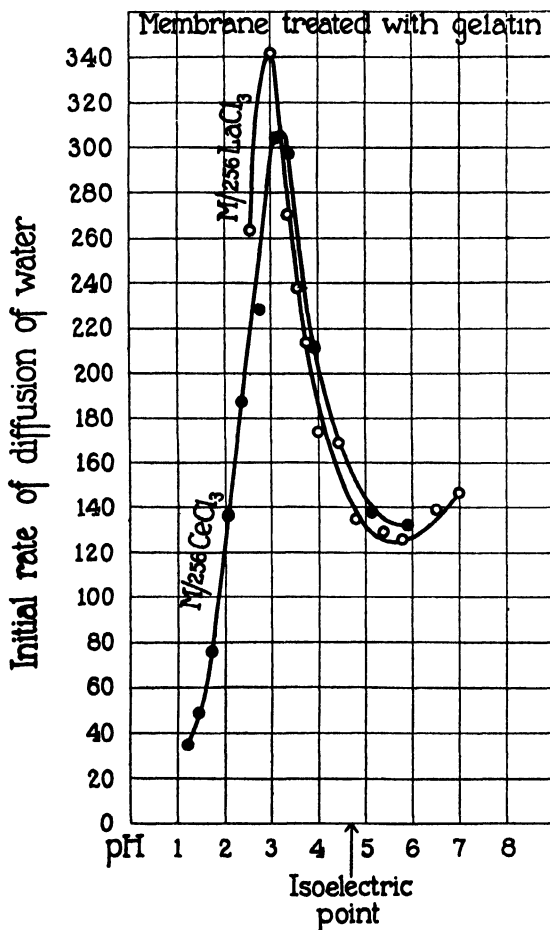
FIG. 5. Although M /256 solutions of La and Ce charge the membrane positively on the alkaline side of the isoelectric point of gelatin, their attraction for water rises very steeply on the acid side of the isoelectric point of gelatin.

point of gelatin the trivalent cation makes the membrane positive by combining with gelatin and forming a salt; *e.g.*, La gelatinate. But, in addition, the salts of trivalent cations attract the water, and the more the higher their concentration until a certain maximum is reached, as shown in Fig. 4. On the acid side from the isoelectric point the protein film cannot react chemically with the La or Ce and the membrane is charged positively through the influence of the
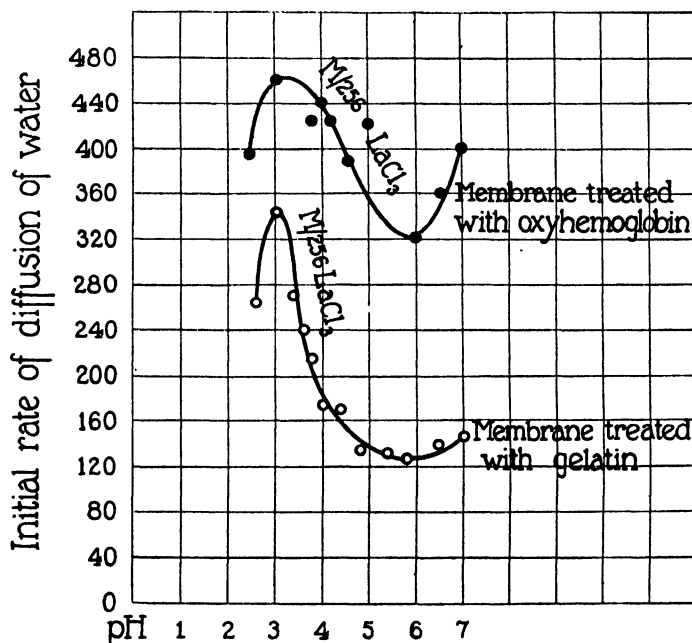
FIG. 6. Showing that the critical point for this steep rise varies with the isoelectric point of the protein with which the membrane has been treated.

acid. In this case the trivalent cation acts apparently by increasing the density of charge of the positive membrane considerably, as was to be expected.

In order to test this idea further, membranes treated with oxy-hemoglobin instead of with gelatin were tried. The isoelectric point of oxyhemoglobin is at pH = 6.8. In this case the steep rise in the curve with LaCl₃ should be nearer this value than 4.7. The curves

of Fig. 6 show that this is the case.   It may be mentioned incidentally that the initial rate of diffusion of water into solutions was greater when the membranes had been treated with oxyhemoglobin than when they had been treated with gelatin.   We shall return to this phenomenon in another connection.
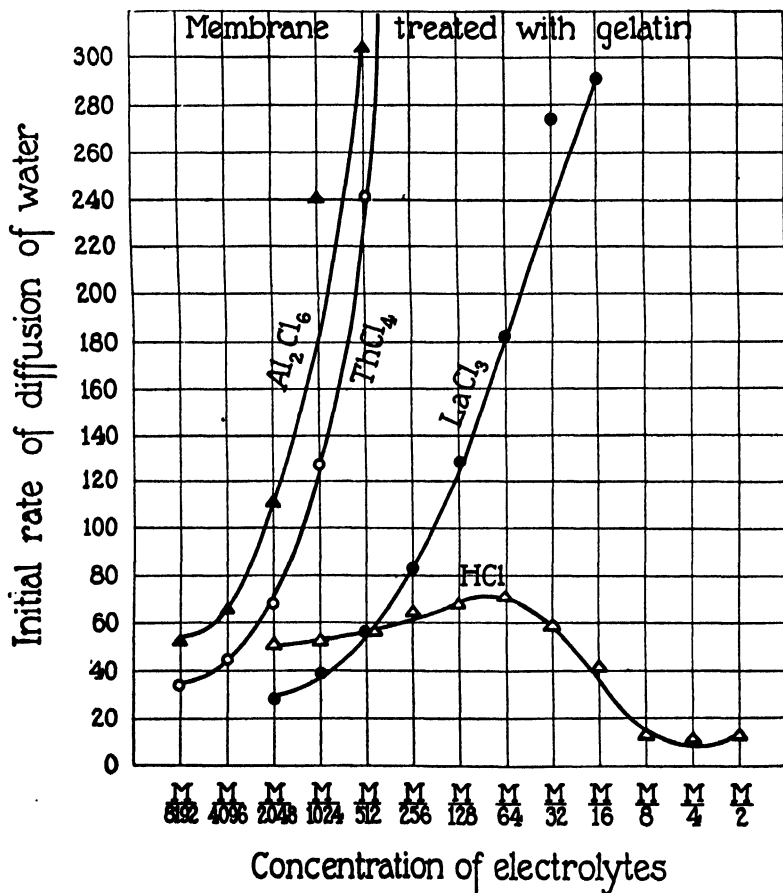
FIG. 7. Showing that the attraction of salts with trivalent cations for water is considerably greater than the attraction of acid (HCl) for water, although acids are very efficient in making the membrane positive.

V.

The experiments represented in Figs. 5 and 6 seem to show that the efficiency of ions in causing the reversal of the sign of charge is as great in the case of hydrogen ions as in the case of trivalent cations; while the influence on the rate of diffusion of water is considerably greater in the case of trivalent cations than in the case of hydrogen ions. Fig. 7 permits a comparison of the attraction of water by solutions of equal concentration of HCl, $LaCl_3$, Al_2Cl_6, and $ThCl_4$ through membranes treated with gelatin. On one side of the membrane was the solution, on the other pure water. If we consider only concentrations up to M/32 this attraction is very slight in the case of HCl as compared with $LaCl_3$ or Al_2Cl_6. (It should be stated that in the experiments with HCl the level of the water was considerably higher at the beginning of the experiment than in the other solutions.)

Theoretical Remarks.

The experiments have shown that a reversal of the sign of charge of a collodion membrane can be brought about by acids and by "neutral" salts with trivalent cation after the inside of the membrane has been in contact for an hour or more with a sufficiently strong solution of a protein whereby apparently a protein film is formed on the inside of the membrane. Collodion membranes not treated are always negatively charged in contact with water, no matter whether or not hydrogen ions and trivalent cations are present in sufficient concentration. Collodion membranes when treated with a protein are also negatively charged when the reaction of the solution is on the alkaline side of the isoelectric point of the protein and when the solution is free from trivalent or tetravalent cations. In this case the protein exists in the form of a metal proteinate dissociating into a positive metal ion, the protein adhering to the inside of the collodion membrane. This might suggest that the membrane becomes negative in contact with water on account of these metal ions dissolving in water, while the solid film of protein anions adhering to the membrane is negatively charged. When the hydrogen ion concentration is raised sufficiently to cause the transformation of the protein

film into a protein-acid salt the reversal in the sign of charge of the membrane would be easily intelligible since the protein-acid salt dissociates into a positive protein cation forming the surface film of the membrane, and an anion which goes into solution.

A difficulty arises, however, if we try to explain the reversal of the sign of charge of the membrane by trivalent cations on the alkaline side of the isoelectric point of the protein. In this case the protein film consists of La or Ce proteinates which are practically insoluble. Practically no dissociation into a negative protein anion and a trivalent cation would be supposed to occur and it seems not possible to state why an insoluble La proteinate should assume a positive charge when in contact with water. As long as this fact is not explained it remains doubtful whether the tentative explanation just given for the acid reversal is correct or complete. It also remains to be explained why the collodion membrane not treated with a protein always assumes a positive charge regardless of the hydrogen ion concentration or the concentration of trivalent cations. It might be argued that the collodion membrane differs from proteins in not being an amphoteric electrolyte and in not being able to combine with trivalent cations. Another possibility must, however, be considered; namely, that the hydrogen ions and trivalent cations influence the surface electrical potential of the solution and that the combined effects of these ions on the surface electrical potential of the membrane and of the liquid determine the phenomena described in our papers.

SUMMARY.

1. Trivalent cations cause a collodion membrane covered with a protein film to be charged positively while they do not produce such an effect on collodion membranes not possessing a protein film. The same had been found for the reversal of the sign of charge of the membrane by acid.

2. This reversal in the sign of charge of the membrane by trivalent cations occurs on the alkaline side of the isoelectric point of the protein used; while the reversal by acid occurs on the acid side of the isoelectric point.

3. The reversal seems to be due to or to be accompanied in both cases by a chemical change in the protein. The chemical change which occurs when the hydrogen ions reverse the sign of charge of the protein film consists in the formation of a protein-acid salt whereby the H ion becomes part of a complex protein cation; while the chemical change which occurs when trivalent cations reverse the sign of charge of the protein film consists in the formation of an insoluble and therefore sparingly or non-ionizable metal proteinate.

their radius is as follows: *The efficiency of anions increases directly and that of cations inversely with the radius of the·ion.* By radius of the ion we mean the distance between the positive nucleus of an ion and its outermost ring or shell of electrons. This term has a definite meaning in the case of monatomic ions.

The method of experimentation is the same as in the preceding publications. The solution of the electrolyte was put into collodion flasks of about 50 cc. content, which had previously been filled over night with a 1 per cent gelatin solution. The gelatin solution was then carefully washed out with warm water. The membrane retained on its inside a very thin film (probably of only one molecule in thickness) of gelatin. This was done to enable us to reverse the sign of charge of the membrane with dilute acid; when the hydrogen ion concentration is below 2×10^{-5} N the membrane is negatively (and the watery phase positively) charged; while when the hydrogen ion concentration is above this value the membrane assumes a positive and the water a negative charge. The membranes used in the experiments to be described had therefore all been treated with gelatin.

The collodion flask was closed with a rubber stopper, through which a glass tube with a bore of about 2 mm. in diameter was pushed inside the flask. The latter was filled with the solution of electrolyte and suspended in a beaker with distilled water having the same hydrogen ion concentration as the solution of electrolyte. The glass tube served as a manometer to indicate the rate at which water diffused from pure water into the solution through the collodion membrane. The temperature was kept constant at 24°C. We shall treat the influence of ions on negatively and positively charged membranes separately.

II. Negatively Charged Membranes.

When an electrical double layer is formed at the boundary of a collodion membrane (previously treated with gelatin as described) the membrane assumes a negative and the watery phase a positive charge as long as the hydrogen ion concentration is below 2×10^{-5} N and no trivalent or tetravalent cations are present. If the hydrogen ion concentration exceeds 2×10^{-5} N and becomes 10^{-4} N the sign of charge on the two layers is reversed. This was proved by experi-

ments on electrical endosmose.[1] We will first describe experiments in which the membrane is negatively and the watery phase positively charged.

We have shown in a previous paper that anions accelerate and cations depress the rate of diffusion of water through negatively charged membranes.[2] Fig. 1 shows the relative influence of the anions of three potassium salts, KCl, KBr, and KI on the rate of diffusion of water through collodion membranes. The ordinates
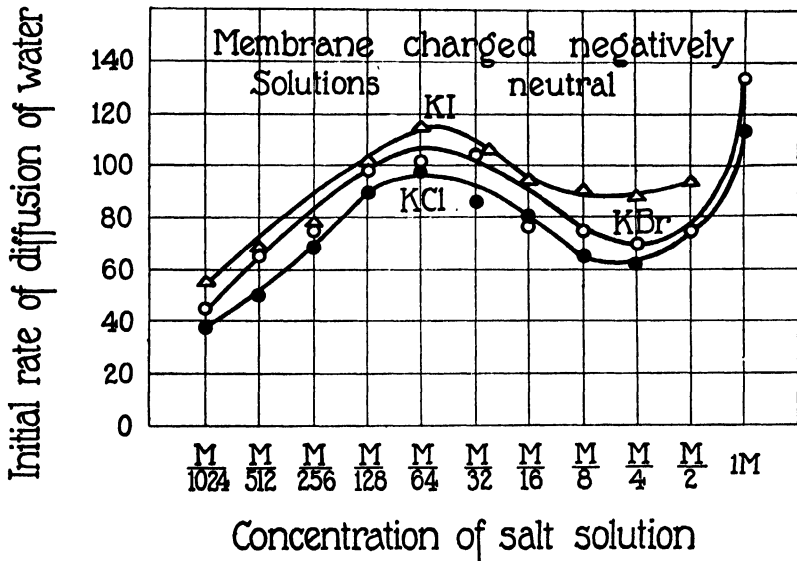
FIG. 1. The rate of diffusion of water through a negatively charged membrane (from the side of pure water to solution) and the density of the charge of the latter are increased by monovalent, monatomic anions in the order I > Br > Cl. Solutions almost neutral (pH about 6.0).

indicate the rise of liquid in the manometer in 20 minutes; the abscissæ indicate the concentration of the solution. The salt solutions as well as the pure water separated from the solution by the collodion membrane were almost neutral, having a hydrogen ion concentration of about 10^{-6} N. It is obvious that the accelerating influence of

[1] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 577.
[2] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 173.

anions on the rate of diffusion of water from pure water into the solution through a negatively charged membrane increases in the order I > Br > Cl, the accelerating influence of the I ion being the greatest.
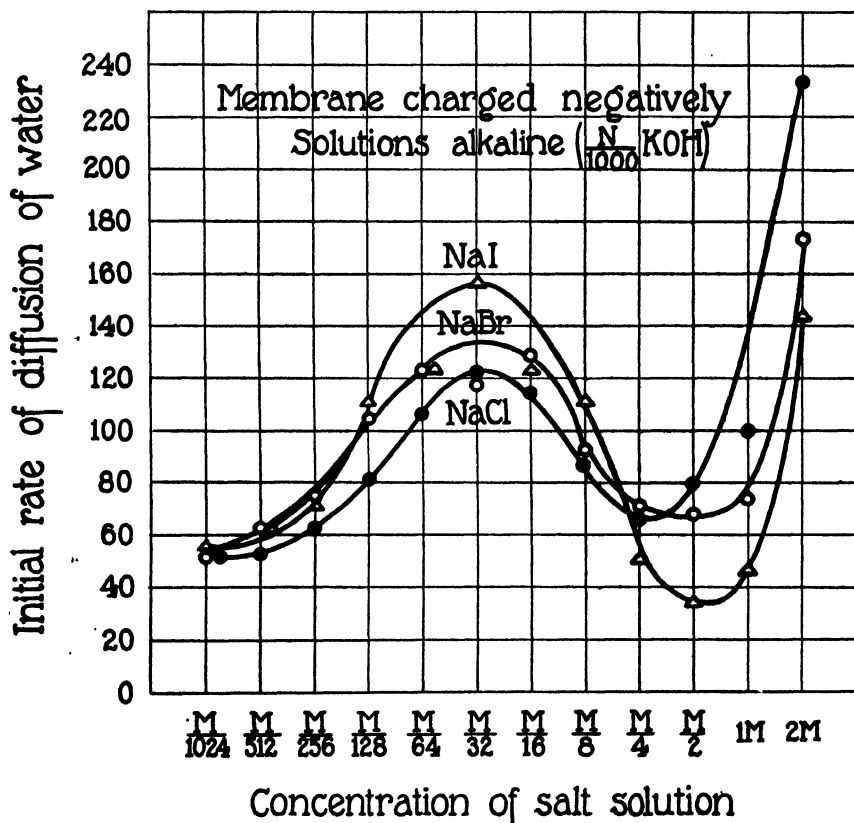
FIG. 2. The same as Fig. 1 except that the solutions and water are alkaline (pH about 11.0).

The same is true when the solution is more alkaline. Thus in Fig. 2 NaCl, NaBr, and NaI were dissolved in a N/1,000 solution of KOH instead of in pure water and the outside solution was N/1,000 KOH. The sign of charge of the membrane remained negative, however, and therefore the relative efficiency of the three anions
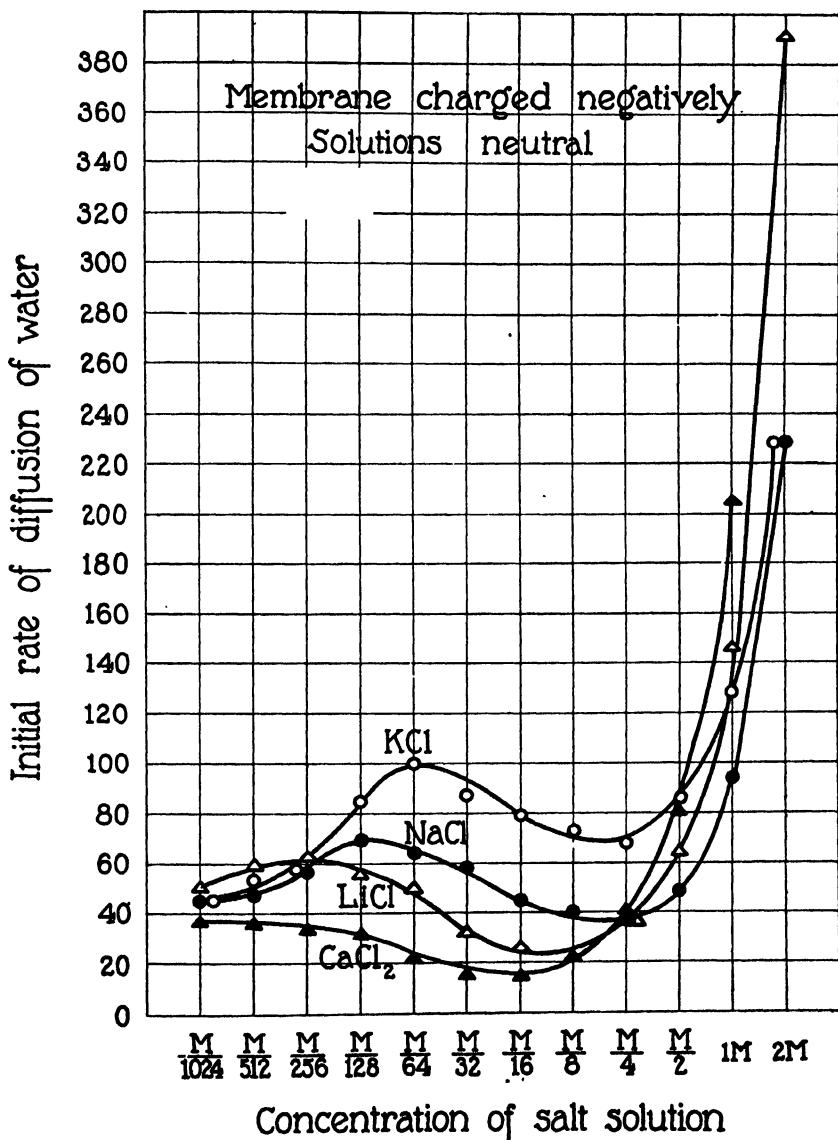
FIG. 3. The rate of diffusion of water through a negatively charged membrane and the density of charge of the latter are depressed by monovalent, monatomic cations in the order Li > Na > K. Solutions neutral (pH about 6.0).

remained the same. The rate of diffusion of water was greater in
the case of solutions of NaI than in the case of NaBr, and greater in
NaBr than in NaCl. Hence the statement is confirmed that when
a membrane is negatively charged the rate of diffusion of water
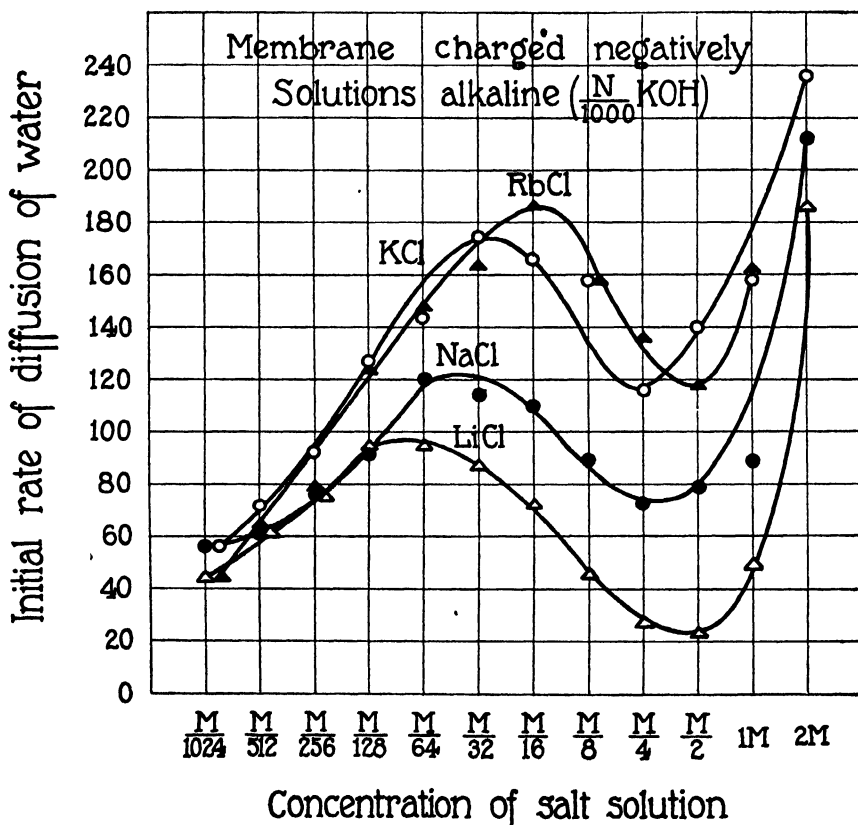
FIG. 4. The same as in Fig. 3 when solutions are alkaline (pH about 11.0).
Order of depressing action of cations on negatively charged membrane Li >
Na > K > Rb.

through the membrane from pure water into solution increases for
the anions I, Br, and Cl in the order I > Br > Cl.

Since this is also the order of the magnitude of the ionic radius of
the three anions, I having the greatest radius, we càn say that the

accelerating effect of monatomic, monovalent anions upon the rate of diffusion of positively charged water through negatively charged membranes from the side of pure water to the side of the salt solution increases directly with the order of magnitude of the radius of the anion.
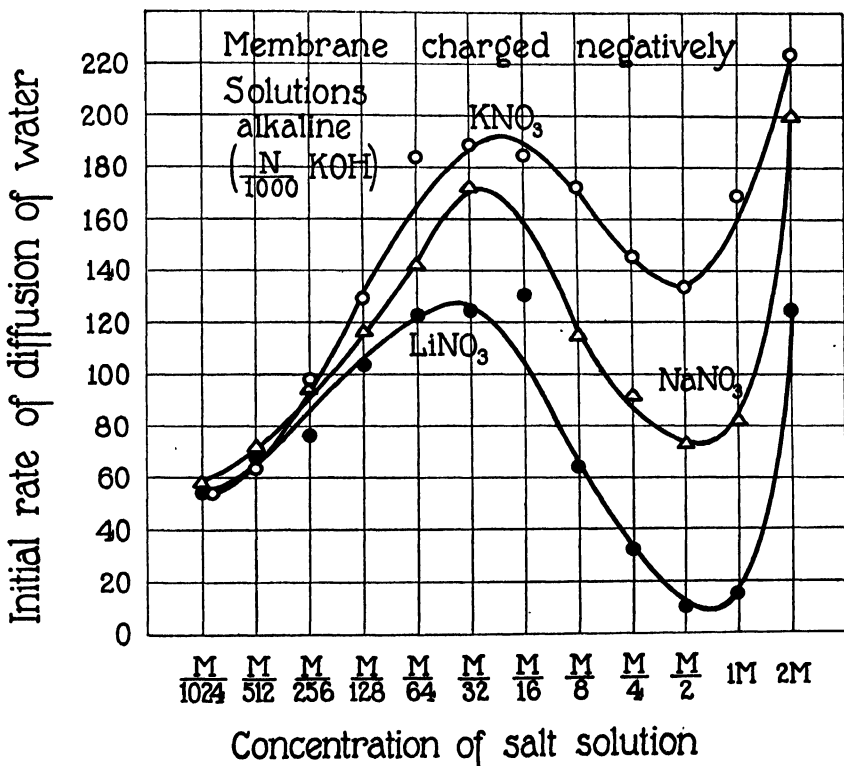
FIG. 5. The depressing order of cations upon negatively charged membranes is the same for NO_3 as for Cl (Fig. 4). Solutions alkaline, order of depressing effect Li > Na > K.

The same relative order of efficiency for the three anions was found also when they were in combination with Li.

While the anions increase the rate of diffusion of positively charged water through a negatively charged membrane cations diminish it and the question arises in what order the depressing effect of the

monovalent cations increases. Fig. 3 shows that in the case of monovalent cations the depressing effect is least in the case of K, and
increases in the following order, K < Na < Li.

In these experiments the solutions were almost neutral (hydrogen
ion concentration about 10^{-6} N). The order of efficiency of the cations
remains the same when the solutions of these salts are made up in
N/1,000 KOH and when the distilled water in the outside solution
is replaced by N/1,000 KOH; since in this case the membrane is
also negatively and the water positively charged. Fig. 4 shows that
the depressing effect of the monovalent, monatomic cations increases
in the order Rb < K < Na < Li, where the depressing effect is
least in the case of Rb, and greatest in the case of Li. This order of
the depressing influence of cations is independent of the anion used
as long as the anion is always the same for the different cations used.
Thus Fig. 5 shows the same order of the depressing action of cations
for nitrates as in Fig. 4 in the case of the chlorides.

Since this is also the order in which the ionic radius of the four
ions diminishes (Rb having the greatest ionic radius) we can make
the following statement.

The depressing effect of monatomic, monovalent cations on the
rate of diffusion of positively charged water through a negatively
charged collodion membrane from the side of pure water to the side
of the salt solution increases inversely with the order of magnitude
of the radius of the cation.

III. Positively Charged Membranes.

It has been shown in preceding publications that when the membrane is positively and the water negatively charged the cations
increase and the anions diminish the rate of diffusion of water from
the side of pure water through the membrane into the solution. In
order to make a membrane, previously treated with gelatin, positively
and. the watery phase of the double layer negatively charged it is
necessary to give the water and the solutions a hydrogen ion concentration of about 10^{-4} N or above. We made the solution acid by
dissolving the salts in 10^{-3} N HNO_3 and the distilled water outside
the collodion bag was also replaced by 10^{-3} N HNO_3. The hydrogen
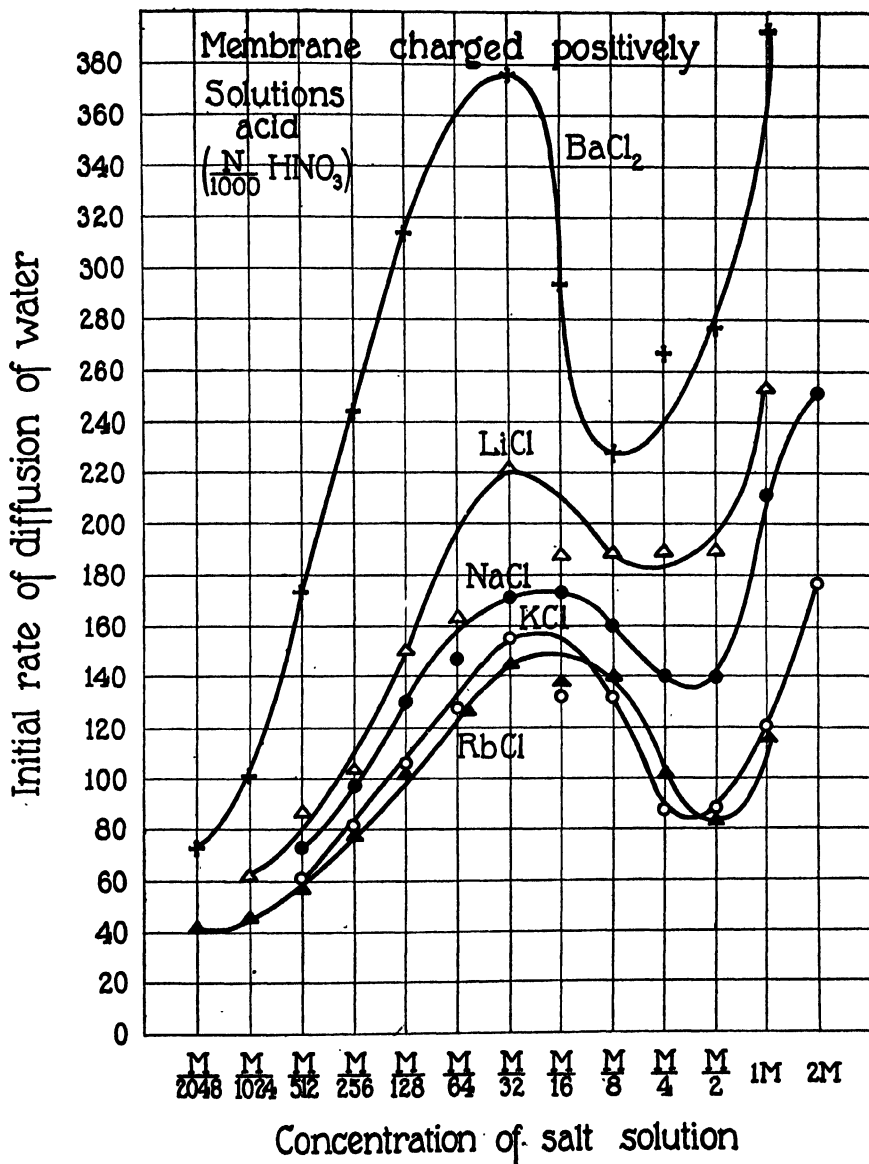
FIG. 6. The rate of diffusion of water through a positively charged membrane and the density of charge of the membrane are increased by the monovalent, monatomic cations in the order Li > Na > K > Rb. Solutions acid (pH about 3.0).

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ion concentration of the solution inside the collodion flask and the
water surrounding the collodion flask had therefore the same hydrogen
ion concentration of about 10^{-8} N.   Fig. 6 shows the order of influence
of different cations upon the rate of diffusion of water through the
membrane into the solution.   The anion is always the same; namely,
Cl.   The order of efficiency of the cation series increases in the order
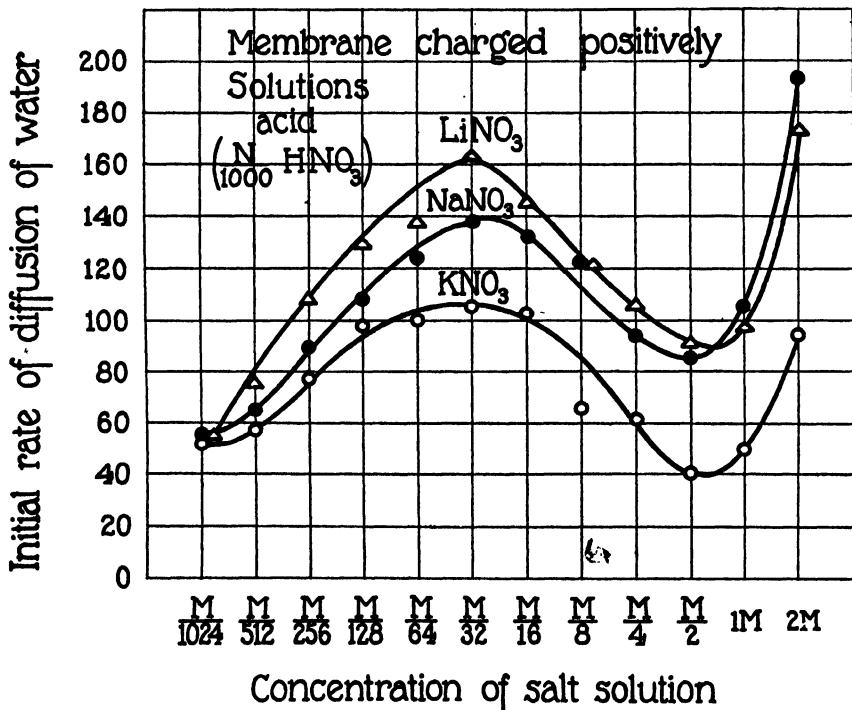
FIG. 7. The same order of efficiency of cations if the anion is NO_3.

Rb < K < Na < Li; *i.e.*, the efficiency of monovalent, monatomic
cations increases inversely with the magnitude of their radius.   This
order is the same regardless of the anion.   Thus Fig. 7 shows that
the order is the same when Cl is replaced by NO_3.

The anion depresses the rate of diffusion of negatively charged
water through the membrane into the solution when the membrane
is positively charged.   Fig. 8 shows that this depressing effect of

different potassium salts increases in the order I > Br > Cl, where the depressing effect of I is greatest.

This order is the same regardless of the nature of the cation. Thus Fig. 9 shows the same order of depressing effect of the anion on the rate of diffusion of water through positively charged membranes in the case of barium salts.
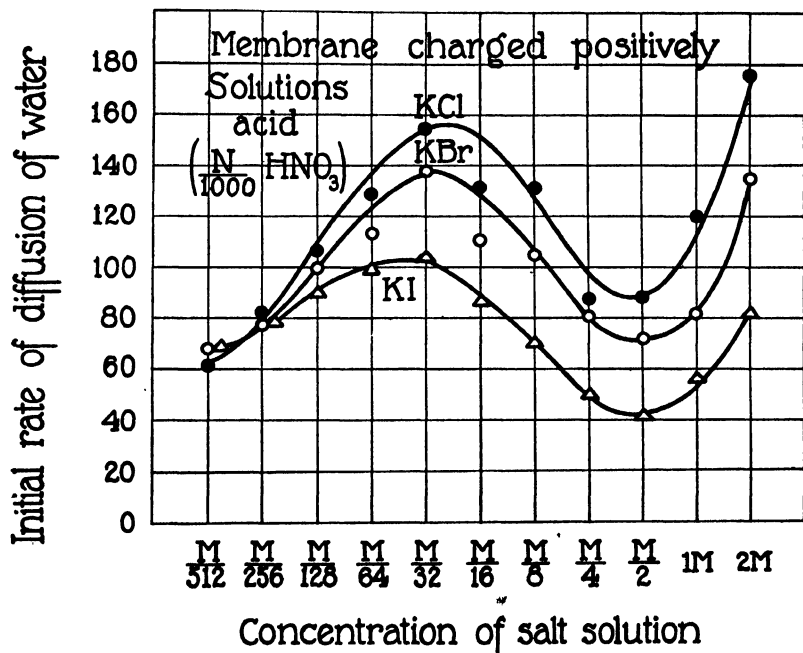
FIG. 8. The rate of diffusion of water through positively charged membranes (pH about 3.0) and the density of charge of membrane are depressed by anions in the order I > Br > Cl.

If we combine the results of the experiments on positively and negatively charged membranes we can express them in the following form.

The accelerating and depressing effect of monatomic, monovalent ions on the rate of diffusion of water through a collodion membrane from the side of pure water to the side of the solution increases for anions directly and for cations inversely with the magnitude of their radius.
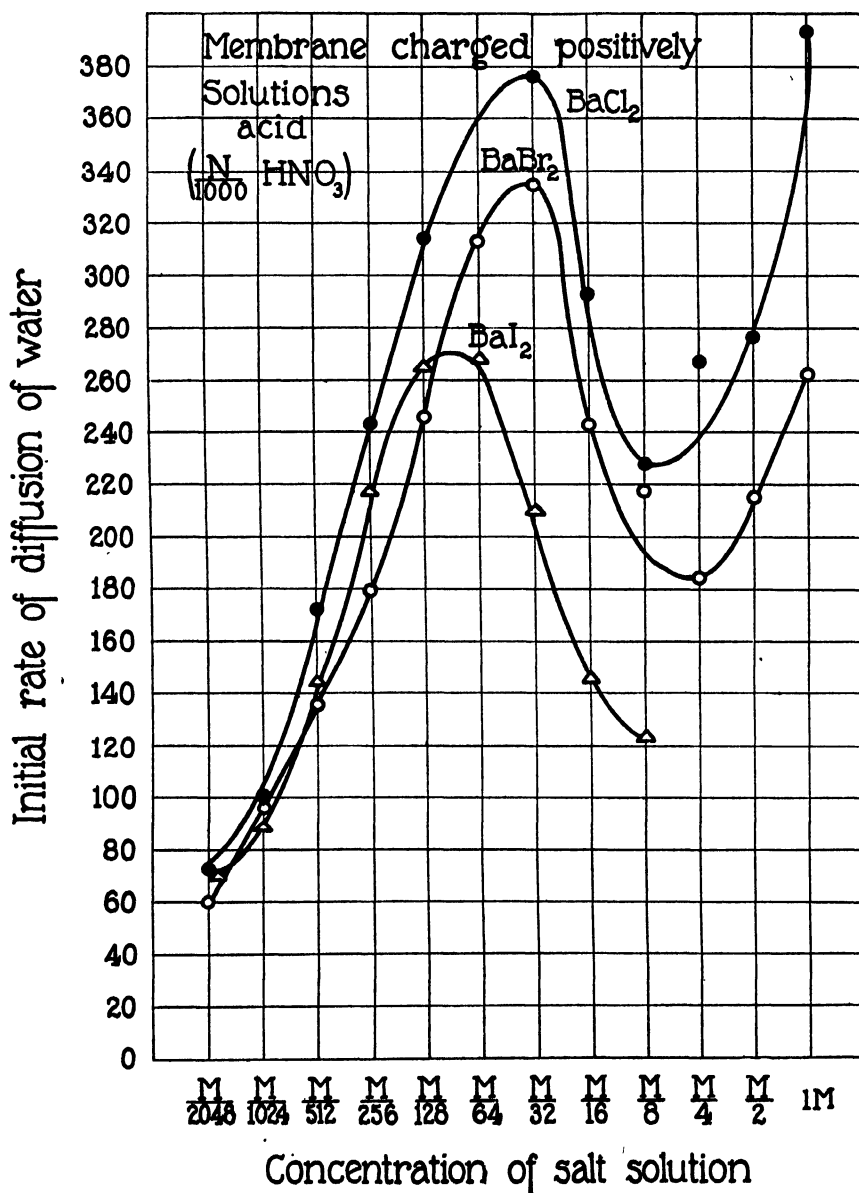
FIG. 9. The same as Fig. 8 for a different cation.

IV. Theoretical Considerations.

Kossel[3] has suggested that for chemical reactions of atoms we may replace the atoms by simplified models consisting only of the charges of the positive nuclei in the center and the ring of valency electrons. The distance between the positive nucleus and the outermost ring or shell of electrons we call the radius of the atom or of the ion. While a monatomic, monovalent cation has one excess positive charge in its nucleus a monatomic, monovalent anion has one excess negative charge in its periphery. Although these positive and negative charges are alike, the relative electrostatic action of ions with the same charge must vary with the radius. Cations act through the excess positive charge on the nucleus, and the electrostatic action of the nucleus must become the greater the smaller its radius; *i.e.*, the nearer the positive nucleus can get to the body on which it is supposed to act. This explains why the accelerating as well as the depressing efficiency of a cation on the rate of diffusion of water through the membrane increases *inversely* with the radius of the cation; namely, in the order Rb < K < Na < Li, where Rb has the minimal effect.

When the nucleus has more than one excess charge the accelerating as well as the depressing efficiency increases with the valency, since *ceteris paribus* the electrostatic action of a cation must increase with the number of excess charges of its nucleus.

We understand also why the accelerating as well as the depressing efficiency of an anion increases *directly* with its radius, since the electrostatic effect of a monovalent anion on an outside body is determined by its excess electron and hence will be the greater the greater the distance between the valency electron and its positive nucleus.

It is also obvious why the efficiency of an anion should increase with its valency; *i.e.*, with the number of valency electrons it contains in excess of its nuclear charges.

We can only speak of ionic radius in the case of monatomic ions like K or Cl; when, however, an ion consists of more than one atom, *e.g.* NO_3, CNS, we have more than one positive nucleus and each nucleus has its own rings or shells of electrons. The term ionic

[3] Kossel, W., *Ann. Physik*, 1916, xlix, 229.

radius loses its meaning in this case unless we substitute for such polyatomic ions a monatomic model with one nucleus, one ring of valency electrons and a radius calculated in such a way as to render the electrostatic effect of the monatomic model equal to that of the polyatomic ion which it is supposed to represent.

The rule at which we arrived, namely that the efficiency of the anions increases directly with their ionic radius while the efficiency of the cations increases inversely with their ionic radius, is probably of general applicability in physical chemistry as well as in physiology, wherever the efficiency of ions depends on their electrostatic action.

There are numerous observations in the physiological literature which indicate an inversion of the order of efficiency of monovalent cations when the reaction of the solution changes from basic to acid. It is possible that these facts will find their explanation on the basis of our rule; namely, that on one side of the neutral (or in certain cases the isoelectric) point the effect observed is increased by the cation and inhibited by the anion, while on the other side it is increased by the anion and inhibited by the cation. Unfortunately the facts given are often too incomplete to test this idea and the experiments are generally done with such high concentrations of electrolytes that it is doubtful whether they can be used for any conclusions concerning the specific effects of ions.

SUMMARY.

1. It has been shown in preceding papers that when we separate solutions of electrolytes from pure water by collodion membranes the ions with the same sign of charge as the membrane increase while the ions with the opposite sign of charge diminish the rate of diffusion of water from the side of pure water to the side of solution; and that the accelerating and depressing effects of these ions on the rate of diffusion of water increase with their valency.

2. It is shown in this paper that aside from the valency a second quantity of the ion plays a rôle in this effect, namely the radius, which in a monatomic ion means the distance between the central positive nucleus and the outermost ring or shell of electrons of the ion. In monatomic, monovalent anions the radius increases in the

order Cl< Br< I (being largest in I), while for monatomic, monovalent cations it increases in the order Li< Na< K< Rb (being largest in Rb).

3. It is shown that the accelerating as well as the depressing effect of the anions mentioned increases directly with the order of magnitude of their radius and that the efficiency is greatest in the case of I which has the largest radius; while the accelerating as well as the depressing effect of cations increases inversely with the order of magnitude of their radius, Li with the smallest radius having the greatest efficiency.

4. This is intelligible on the assumption that the action of the ions is electrostatic in character, in the case of cations due to the electrostatic effect of the excess charge of their positive nucleus, and in the case of anions due to the excess charge of their captured electron. The electrostatic effect of the positive nucleus of a cation on the membrane (or any other body) will be the greater the smaller the ionic radius of the cation; and the electrostatic effect of an excess electron will be the greater the further its distance from its own positive nucleus.

5. It is suggested that this rule may possibly include polyatomic, monovalent ions (e.g. NO_3, CNS, etc.) when we replace these polyatomic ions by monatomic models in which the radius is calculated in such a way as to give the model the same electrostatic effect which the polyatomic ion possesses.

6. These conclusions are in harmony with the fact that the efficiency of ions increases also with their valency.

7. It is suggested that these rules concerning the influence of the ionic radius can possibly be demonstrated in other phenomena, depending on the electrostatic effect of ions.

ION SERIES AND THE PHYSICAL PROPERTIES OF PROTEINS. I.

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I. INTRODUCTION.

It had been shown in a preceding paper[1] that the relative influence of ions on the rate of diffusion of water through collodion membranes can be expressed by arranging the ions in two definite series, as follows:

(1) Rb < K < Na < Li < divalent < trivalent cations.

(2) Cl < Br < I < divalent < trivalent anions.

These results are intelligible on the assumption that we are dealing in this case with electrostatic effects of ions, the electrostatic effects of the cations being due to the excess charges of the positive nucleus and the electrostatic effects of the anions being due to the charges of the captured valency electrons. In this case the relative efficiency of monatomic and monovalent cations should increase inversely with the radius of the ion; and the relative efficiency of the monovalent, monatomic anions should increase directly with the radius of the ion. In the cation series the Li ion should be next to the divalent cations, while in the anion series the iodion should be next to the divalent anions.

This order differs from that usually given for the action of ions on the physical properties of proteins. Hofmeister[2] and afterwards Pauli[3] determined the relative order of efficiency of ions for the precipitation of proteins. This order is according to Pauli:

[1] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 673.

[2] Hofmeister, F., *Arch. exp. Path. u. Pharm.*, 1888, xxiv, 247.

[3] Pauli, W., *Beitr. chem. Physiol. u. Path.*, 1903, iii, 225; *Fortschr. naturwiss. Forschung*, 1912, iv, 237.

For cations, Mg < NH$_4$ < K < Na < Li.

For anions, CNS < I < Br < Cl < acetate < tartrate < citrate < phosphate < sulfate < Fl.

The reader will notice that in the cation series the divalent cation Mg is next to NH$_4$ and K, while we should expect it to follow Li in order of efficiency. On the other hand, if the efficiency of the monovalent, monatomic cations increases in the order of K < Na < Li as Hofmeister and Pauli observed we should expect the efficiency of the anions to increase in the order of Cl < Br < I instead of in the reverse order. We must conclude that the phenomena of precipitation of proteins by ions are either not determined by the electrostatic forces of the ion or that other variables enter which are not yet known. This latter possibility exists since the concentration of electrolytes required for precipitation is very high and the writer has shown that the influence of ions causing the anomalous diffusion of water through membranes already ceases to be noticeable in comparatively low concentrations of electrolytes.[4]

It therefore seems advisable to restrict our attention to such influences of ions where they act in low concentrations. This is possible when we compare the effects of low concentrations of different acids and alkalies on the physical properties of proteins. The first one who undertook such a study on a larger scale was Pauli. He recognized that in order to investigate the effect of acids and alkalies on proteins it was necessary to free them first from salts. When Pauli and Handovski[5] added to blood albumin dialyzed for 6 weeks various quantities of different acids they found that all the acids increased the viscosity of the blood albumin but in a different degree. Their results are expressed in curves from which we gather that the relative order of efficiency of various acids tried by them was

HCl > monochloracetic > oxalic > dichloracetic > citric > acetic > sulfuric > trichloracetic acid

where HCl raises the viscosity most, and trichloracetic least. The viscosity was measured by the time of outflow through a viscometer. Pauli assumes, as Laqueur and Sackur[6] had done previously in their

[4] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 173.

[5] Pauli, W., and Handovski, H., *Biochem. Z.*, 1909, xviii, 340.

[6] Laqueur, E., and Sackur, O., *Beitr. chem. Physiol. u. Path.*, 1903, iii, 193.

experiments on casein solutions, that the increase in the viscosity of the protein caused by the addition of acid (or of alkali) is due to an increase in the ionization of proteins, as a consequence of the salt formation between protein and acid or alkali, the protein salt being capable of a greater degree of ionization than the protein not treated with acid or alkali. In order to explain the relative differences in the effect of various acids on the viscosity, Pauli assumes that the protein salts formed with different acids differ in their degree of electrolytic dissociation. He states; e.g., that "the strong trichloracetic acid and the slightly weaker sulfuric acid furnish few protein ions."[7] He also states that the relative efficiency of different anions on the other properties of proteins, as osmotic pressure, precipitation by alcohol, is the same as that found for viscosity.

In his experiments on the influence of acids and alkalies on the osmotic pressure of gelatin solutions the writer had arrived at the conclusion that the results he obtained cannot be expressed in terms of ion series.[8] These experiments showed only an influence of the valency of the anion or cation in combination with the gelatin, but no other influence. Solutions of Li, Na, K, and NH_4 gelatinate of the same pH and the same concentration of originally isoelectric gelatin had the same osmotic pressure. The same was true for Ca and Ba gelatinate; but the osmotic pressure of Ba and Ca gelatinate was only one-half or less than that of Li, Na, K, or NH_4 gelatinate of the same pH and the same concentration of originally isoelectric gelatin. The influence of acids on the physical properties of proteins was still more interesting. Solutions of gelatin chloride, bromide, nitrate, acetate, phosphate, citrate, tartrate, succinate, of the same pH and the same concentration of originally isoelectric gelatin, had approximately the same osmotic pressure, while the osmotic pressure of solutions of gelatin sulfate was only half or less than half of that of gelatin chloride, etc. The osmotic pressure of solutions of gelatin oxalate was almost but as a rule not quite as high as that of gelatin chloride.[8]

This peculiarity found its explanation in an investigation of the combining ratios of these acids with gelatin. · The writer was able to

[7] Pauli, W., *Fortschr. naturwiss. Forschung*, 1912, iv, 243.

[8] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 483, 559.

show that the strong dibasic acid H_2SO_4 combines in equivalent proportions with gelatin while the weaker acids, *e.g.* phosphoric or oxalic, combine in molecular proportions.   At a given pH, *e.g.* pH = 3.5, 1 gm. of originally isoelectric gelatin is in combination with three times as much 0.1 N acid when the acid is H_3PO_4 than when it is HCl; and with almost twice as much 0.1 N acid when the acid is oxalic than when it is HCl; while the ratio of HCl and H_2SO_4 is 1.   It follows from this that the strong dibasic acid H_2SO_4 forms a salt with gelatin in which the anion is divalent, namely SO_4, while the weak tribasic acid H_3PO_4 forms a salt with gelatin in which the anion is monovalent; namely, H_2PO_4 (instead of the trivalent anion PO_4).   Likewise, citric, succinic, and tartaric acids form gelatin salts in which the anion is always monovalent; namely, H-succinate, H-tartrate, etc.   In the case of oxalic acid this is also the case though a slight amount of gelatin salt with divalent anion is probably formed and this might account for the fact that the osmotic pressure of gelatin oxalate is generally slightly less than that of gelatin chloride.[9]

It was also found that $Ca(OH)_2$ and $Ba(OH)_2$ combine with gelatin in equivalent and not in molecular proportion thus showing that the cation of Ca gelatinate and Ba gelatinate is divalent.[8]

All these facts together show that solutions of salts of gelatin with a bivalent ion have an osmotic pressure of one-half or less of that of solutions of salts of gelatin with monovalent ions at the same pH and the same concentration of originally isoelectric gelatin.

This then proves that while the valency of the ion in combination with the gelatin has a strong influence on the osmotic pressure of the gelatin solution differences in the nature of ions of the same valency have either no effect on the osmotic pressure or if they have such an effect it is too slight to be noticeable by our method of experimentation.   We can certainly say that it is impossible to express our observations in terms of the Hofmeister series.   In the Hofmeister series phosphates and chlorides stand at almost opposite ends of the anion series while in our experiments the effects of H_3PO_4 and HCl are identical if we compare the effects at the same pH of the gelatin solutions.   The reason that Pauli arrived at a different conclusion lies

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[9] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 483.

probably in the fact that he compared the effects of the addition of equal quantities of acid to a protein while we compared the effects of different acids at equal hydrogen ion concentrations of the solutions.

II. Action of Weak and Strong Monobasic Acids.

We will first indicate why it is necessary to choose equal hydrogen ion concentrations as a standard of comparison instead of using equal quantities of acid. We always use as standard material isoelectric protein, to which we add enough acid or alkali to bring it to the desired hydrogen ion concentration. In the case of gelatin we proceed usually in this way; we add to 1 gm. of powdered gelatin brought to the isoelectric point (pH = 4.7) small quantities of acid to bring the samples to the desired hydrogen ion concentration, then melt the gelatin, and bring the volume of the solution to 100 cc. by adding H_2O. The pH is then determined. It is hardly necessary to state that it requires greater quantities of weak than of strong acid to bring the gelatin to the same pH on the acid side of the isoelectric point. Fig. 1 gives the quantities of 0.1 N acetic, monochloracetic, dichloracetic, and trichloracetic acids required to bring 1 gm. of isoelectric gelatin in a 1 per cent solution to the same pH. The abscissæ in Fig. 1 are the pH of the gelatin solution resulting from the addition of acid and the ordinates are the numbers of cc. of 0.1 N acid which must be contained in 100 cc. of a 1 per cent solution of originally isoelectric gelatin to produce the pH. The curve indicates that the quantity of acid required is the less the stronger the acid. If we now measure the osmotic pressure of the solutions of the four gelatin acetates (all 1 per cent in regard to the originally isoelectric gelatin) and if we plot the curves with the values for the osmotic pressure as ordinates and the pH as abscissæ, the curves for all four gelatin-acetate salts are practically identical (Fig. 2). All four curves have a minimum at the isoelectric point pH = 4.7; they all rise identically with a diminution in pH (i.e. a rise in hydrogen ion concentration); all reach their maximum at a pH of about 3.5, and all drop almost as steeply with a further diminution of pH as they rise on the ascending side of the curve. The drop will not be discussed in this paper.

The slight differences in the maximal height of the four curves are within the limits of the accuracy of these experiments, and the same or even greater variations may be observed when the same experiment is repeated several times with the same acid.   The maximal values of osmotic pressure reached in these experiments at pH 3.5 are the same as those reached with gelatin salts of any other monovalent anion, Cl, Br, NO$_3$, phosphate, citrate, succinate, etc.
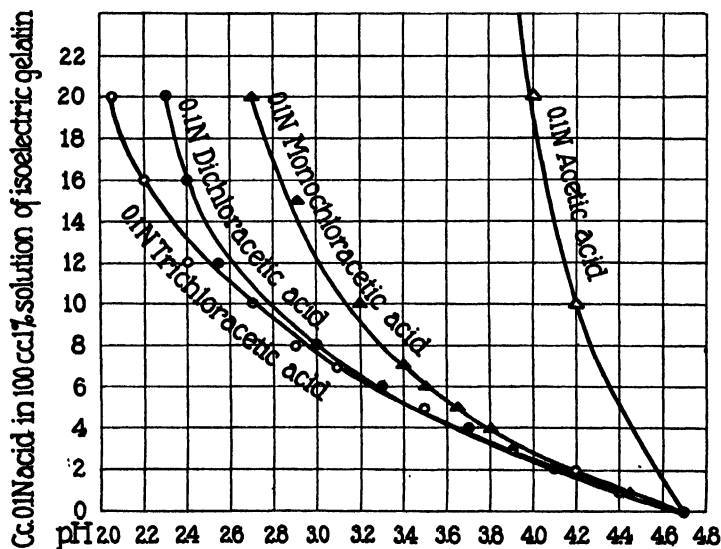
Fig. 1. The ordinates represent the number of cc. of 0.1 N acetic, mono-, di-, and trichloracetic acids required to bring 1 gm. of isoelectric gelatin to the pH indicated by the abscissæ.   Enough H$_2$O was added to bring the gelatin-acid solution to a volume of 100 cc.

It follows from this that the conclusions which are based upon a comparison of the quantities of the acid added instead of upon the pH of the protein solutions cannot be correct.

The reason why we get identical curves when we plot the osmotic pressures as ordinates over the pH as abscissæ seems to be as follows.

When we add a definite small quantity of acid to 1 gm. of isoelectric gelatin, melt, and bring the volume of the solution to 100 cc. by adding H$_2$O, part of the acid will combine with gelatin and part will remain

free in the solution.   The more acid we add, the greater the amount
of isoelectric gelatin transformed into gelatin-acid salt.   We assume
that there is a definite equilibrium between the hydrogen ion concen-
tration of the solution, the amount of gelatin-acid salt formed, and
isoelectric gelatin.   If this view is correct, it follows that at the same
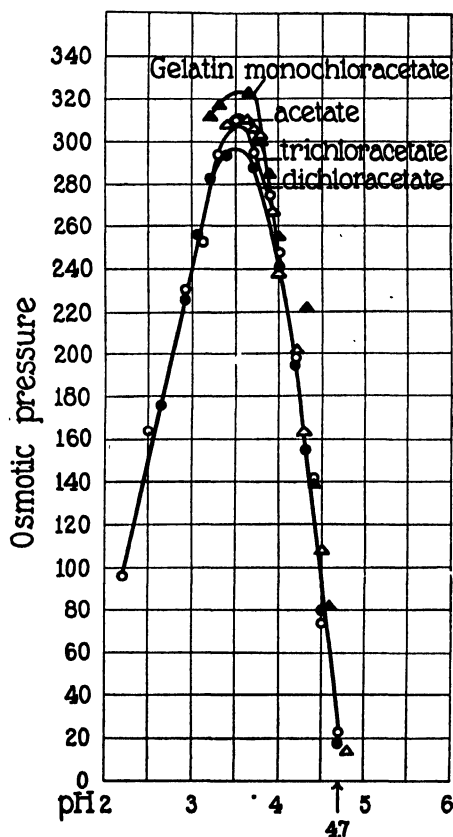
Fig. 2. The ordinates indicate the osmotic pressure (in mm. of the height of
a column of the gelatin solution) of 1 per cent solutions of originally isoelectric
gelatin which have been brought to different pH by the addition of the acids used
in Fig. 1.   The abscissæ are the pH.   The curves are practically identical, the
slight differences at the summit being inside the range of the variation found for
the same acids.

pH and with the same concentration of originally isoelectric gelatin all mixtures of acid and gelatin solution must have the same concentration of gelatin-acid salt; and if this is the case they must all have the same osmotic pressure if the valency of the anion is the same, since the osmotic pressure is almost entirely determined by the gelatin-acid salt, that of the isoelectric gelatin being very low. This conclusion is in harmony with the view expressed by Laqueur and Sackur, Pauli, and others. The writer differs from Pauli only in regard to the statement that the different acids, *e.g.* HCl, H_3PO_4, tartaric, acetic, and trichloracetic, have different effects on the physical properties of proteins.

III. *Combining Ratios of Acids and Alkalies with Crystalline Egg Albumin and the Osmotic Pressure of the Albumin Solutions.*

Crystalline egg albumin was prepared under Dr. Northrop's supervision according to Sörensen's method,[10] and crystallized three times. The only difference in procedure was in the dialysis. Instead of putting the water under negative pressure as was done by Sörensen, pressure was put on the egg albumin by attaching a long glass tube full of water to the dialyzing bag so that the solution was under about 150 cm. water pressure during dialysis. This was necessary to avoid too great an increase in volume. The same stock solution of albumin served for all the experiments and was diluted before the experiment to a 1 per cent solution. The concentration of ammonium sulfate left in the solution was between $M/1,000$ and $M/2,000$. The pH of the stock solution was about 5.20. By adding about 1 cc. of 0.1 N HCl to 100 cc. of a 1 per cent solution of this albumin the solution was brought to the isoelectric point of the egg albumin, which is according to Sörensen at pH = 4.8.

The 1 per cent solutions were made up with different quantities of acid (or alkali) and the pH of the albumin solution was determined electrometrically.[11]   In Fig. 3 are plotted the curves in which the pH are the abscissæ and the cc. of 0.1 N acid required to obtain the various

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[10] Sörensen, S. P. L., *Compt. rend. trav. Lab. Carlsberg*, 1915–17, xii, 1.

[11] The colorimetric determination which gives fairly good results in the case of gelatin is unreliable in the case of egg albumin.

pH as ordinates. The curves represent these values for four acids, HCl, H₂SO₄, H₃PO₄, and oxalic acid. Beginning with the lowest curve we notice that the curve is the same for 0.1 N HCl and 0.1 N H₂SO₄, since both are strong acids; or, in other words, H₂SO₄ combines
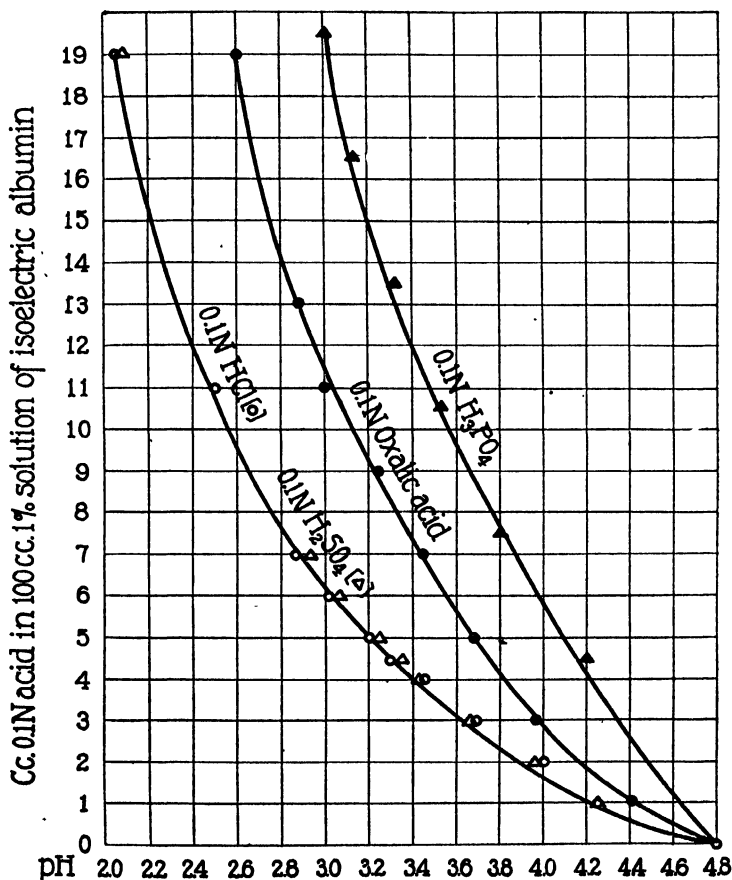
FIG. 3. The ordinates represent the number of cc. of 0.1 N HCl, H₂SO₄, oxalic, and phosphoric acids required to bring 1 gm. of isoelectric crystalline egg albumin to the pH indicated on the axis of abscissæ. Enough H₂O was added to bring the solutions of albumin and acid to a volume of 100 cc. For the same pH the ordinates for HCl, H₂SO₄, and phosphoric acid are approximately as 1:1:3. The ratio of HCl : oxalic acid is a little less than 1:2.

in equivalent proportions with egg albumin. The curve for H_3PO_4 is the highest curve and if we compare the values for H_3PO_4 with those for HCl (or H_2SO_4) we notice that for each pH the ordinate for H_3PO_4 is as nearly three times as high as that for HCl as the accuracy of our experiments permits. This means that under the conditions of our experiments phosphoric acid combines with albumin in molecular proportions and that the anion of albumin phosphate is the mono-valent anion H_2PO_4.
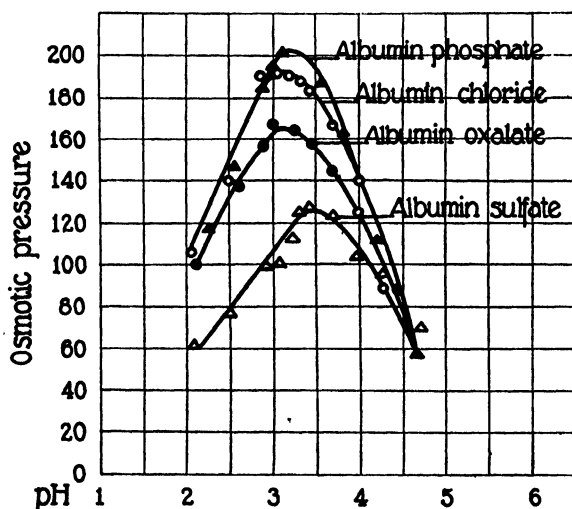
FIG. 4. Osmotic pressure of different albumin-acid salts. The ordinates indicate the osmotic pressure (in mm. of 1 per cent albumin solution); the abscissæ are the pH. All solutions are 1 per cent in regard to isoelectric albumin. The curves for albumin chloride and albumin phosphate are identical.

The values for oxalic acid are for pH above 3.2 not quite twice as high as those for HCl, indicating that for these values of pH oxalic acid combines to a greater extent in molecular and only to a smaller extent in equivalent proportions with albumin.

If egg albumin behaves like gelatin we should expect that the curves of osmotic pressure for albumin phosphate and albumin chloride when plotted as a function of the pH should be identical, since the anion is in both cases monovalent; that the curve for albumin sulfate should

be considerably lower since the anion SO_4 in combination with albumin is bivalent; while the curve for the osmotic pressure of albumin oxalate should be between the curves for albumin chloride and albumin sulfate, but much nearer the chloride than the sulfate curve. Fig.
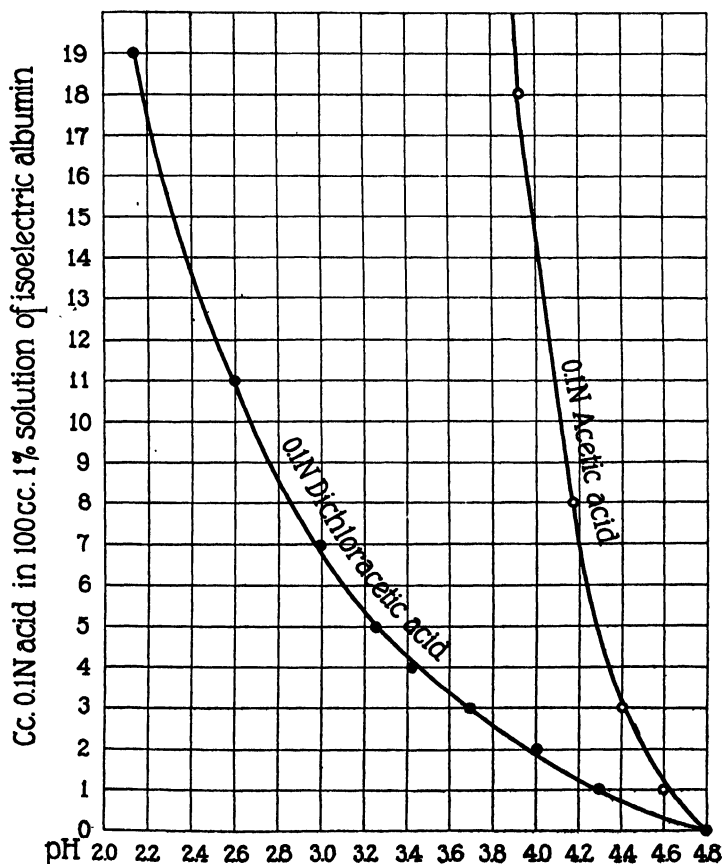
FIG. 5. Curves expressing the cc. of 0.1 N acetic and dichloracetic acids required to bring 1 gm. of isoelectric albumin to different pH. .

4 giving the curves of the osmotic pressures of the four albumin salts shows that this expectation is fulfilled.

Fig. 5 gives the curves for the combining ratios of acetic acid and dichloracetic acid with isoelectric albumin, showing the same differ-

ence as the corresponding curves for gelatin in Fig. 1. Fig. 6 gives the curves for the influence of the two acids upon the osmotic pressure of 1 per cent solutions of originally isoelectric albumin. The two curves are identical and are also identical with those of albumin chloride and albumin phosphate in Fig. 4, thus confirming our theory.

In Fig. 7 are given the curves for combining ratios of NaOH, $Ca(OH)_2$, and NH_4OH with isoelectric albumin. The curve for $Ca(OH)_2$ is identical with that for the strong base NaOH, indicating that $Ca(OH)_2$ combines with egg albumin in equivalent proportions.
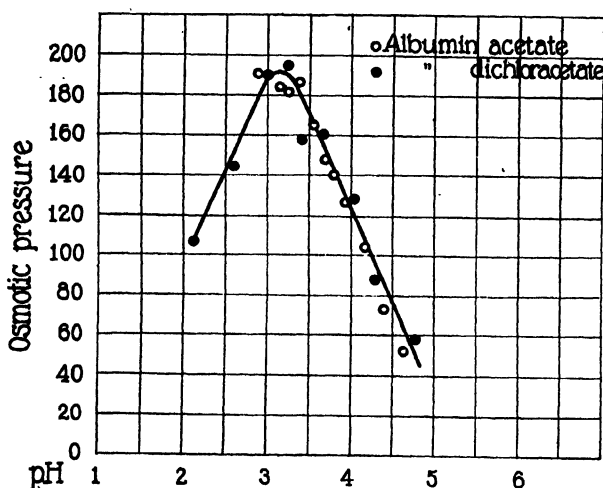
FIG. 6. Curves of osmotic pressure of solutions of albumin acetate and albumin dichloracetate. The curves for both acids are identical.

Fig. 8 shows that the curve for the osmotic pressure of Ca albuminate is only one-half as high as that of Na albuminate as was to be expected. The curve for NH_4 albuminate is identical with that for Na albuminate, which was to be expected since the NH_4 is monovalent.

The results with albumin are therefore identical with those obtained in the case of the corresponding gelatin salts. The result that gelatin phosphate and albumin phosphate behave like gelatin chloride and albumin chloride may be considered as a crucial test against the colloidal conception of ion effects on proteins and in favor of a purely chemical theory.
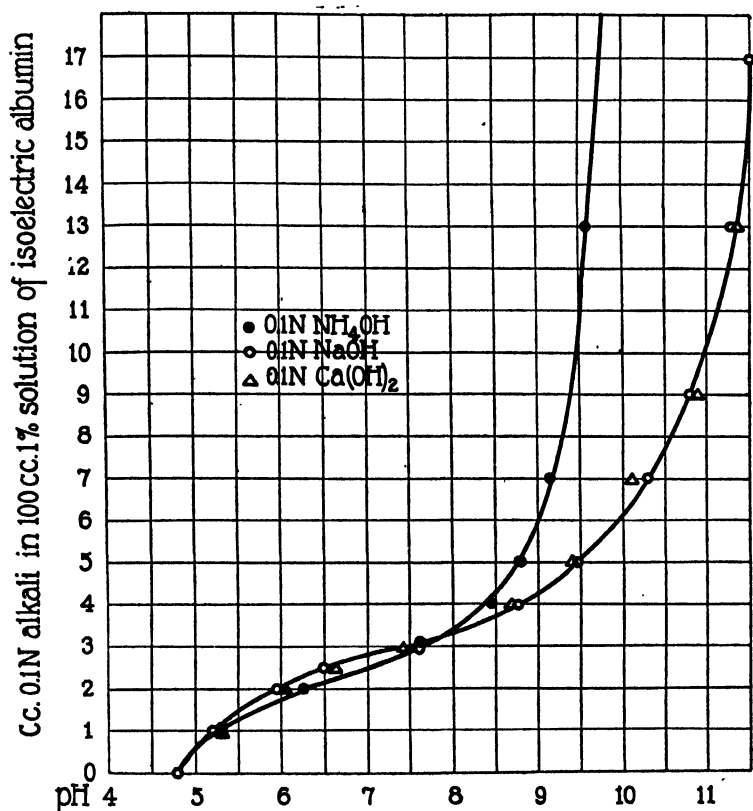
FIG. 7. Curves representing the number of cc. of 0.1 N NH₄OH, NaOH, and Ca(OH)₂ required to bring 1 gm. of isoelectric, crystalline egg albumin to different pH. The curves for NaOH and Ca(OH)₂ are identical.
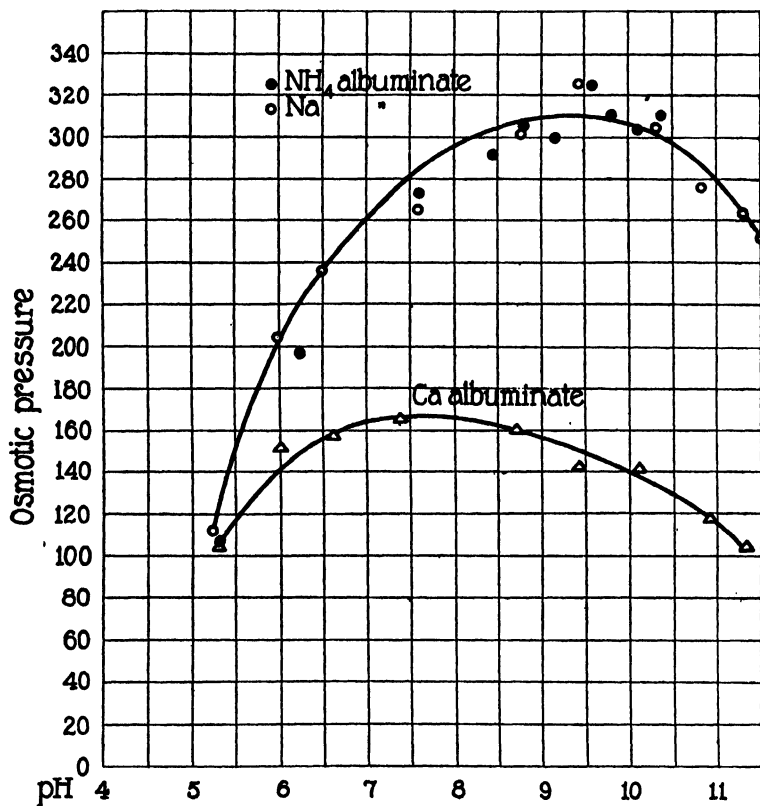
FIG. 8. Curves of the osmotic pressure of NH₄, Na, and Ca albuminate at different pH. The curves for NH₄ and Na albuminate are practically identical.

IV. Combining Ratios of Acids and Bases with Gelatin and the Viscosity of Gelatin Salts.

Since Pauli's ion series was based primarily on the influence of acids or their anions on the viscosity of protein solutions it seemed necessary to find out whether or not viscosity measurements confirm the conclusions at which we arrived on the basis of osmotic pressure experiments on gelatin and egg albumin. A few remarks concerning our method are required.

The gelatin is first rendered isoelectric in the following way. 25 gm. of powdered gelatin of pH about 7.0 are put into 1 liter of M/128 acetic acid for 30 minutes at 10°C., after which time the acetic acid is renewed and left in contact with gelatin again for 30 minutes at 10°C. The acid is then decanted and replaced with distilled water of about 5°C. The mixture is filtered in a Buchner funnel through muslin, employing slight suction. The gelatin is then washed about six times with 100 cc. of distilled water of 5° each, and is made into a 5 per cent solution which serves as a stock solution. The pH of this solution is about 4.7, or, in other words, the gelatin is isoelectric.

Some of the stock solution is heated to 45° and made up to a 2 per cent solution in quantity sufficient for a day's experiments. This 2 per cent solution is kept during the day at 24°C. To 50 cc. of this solution is added the desired acid or alkali in sufficient quantity and then the volume is raised to 100 cc. by the addition of enough distilled water. This 1 per cent solution is then rapidly brought to a temperature of 45°, kept there for 1 minute, and is then rapidly cooled to 24°. The solution is stirred constantly during the heating and cooling. The viscosity is measured immediately after the solution is cooled to 24°C. The measurements were all made at 24°C. by using the time of outflow through a viscometer. The time of outflow of distilled water through an Ostwald viscometer at 24°C. was exactly 1 minute. Each measurement of viscosity was repeated with the same gelatin solution and the beginning and the end of a series consisted in the measurement of viscosity of isoelectric gelatin. These latter measurements agreed in all experiments within 1 second varying only between 80 and 81 seconds, thus guaranteeing the reproducible character of the experiments.

The results can be given briefly. Fig. 9 gives the curves for the cc. of 0.1 N HNO$_3$, H$_2$SO$_4$, oxalic, and phosphoric acids required to bring 1 gm. of isoelectric gelatin to different pH. The curve shows again that for each pH the number of cc. of 0.1 N acid required are for HNO$_3$, H$_2$SO$_4$, oxalic, and phosphoric acids approximately as 1:1:2:3.
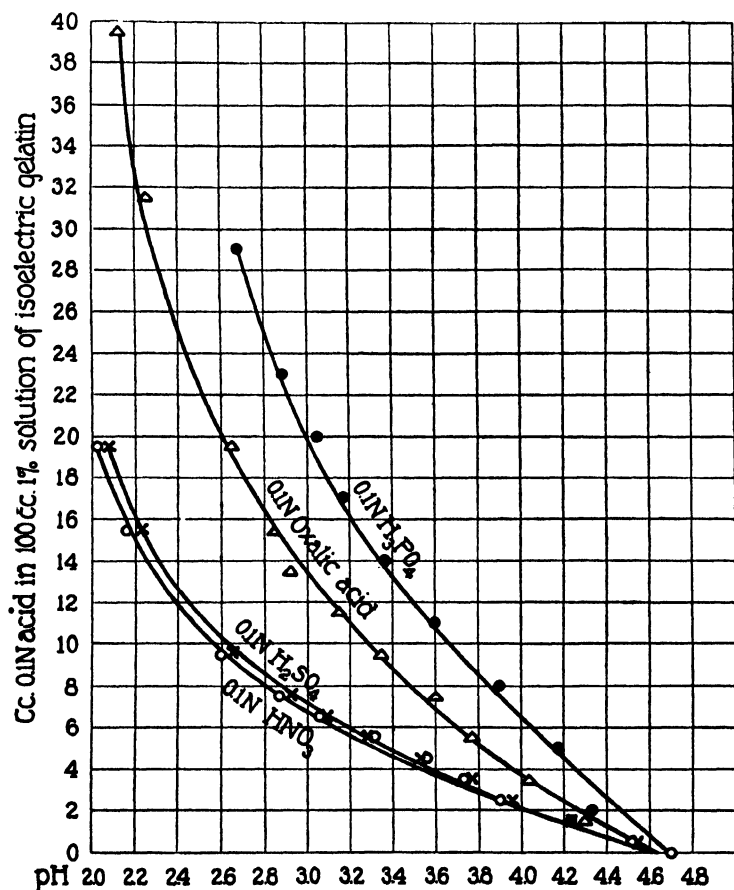
FIG. 9. Curves for the number of cc. of 0.1 N HNO$_3$, H$_2$SO$_4$, oxalic, and phosphoric acids required to bring 1 gm. of isoelectric gelatin to different pH (in 100 cc. of solution). Curves similar to those for egg albumin (Fig. 3). For the same pH the ratio of HNO$_3$, H$_2$SO$_4$, oxalic, and phosphoric acids required is approximately as 1:1:2:3.

Fig. 10 gives the curves for the viscosity of 1 per cent solutions of gelatin chloride, sulfate, oxalate, and phosphate. The abscissæ are the pH, the ordinates the ratio of the time of outflow of the gelatin solutions divided by the time of outflow of pure water. For the sake
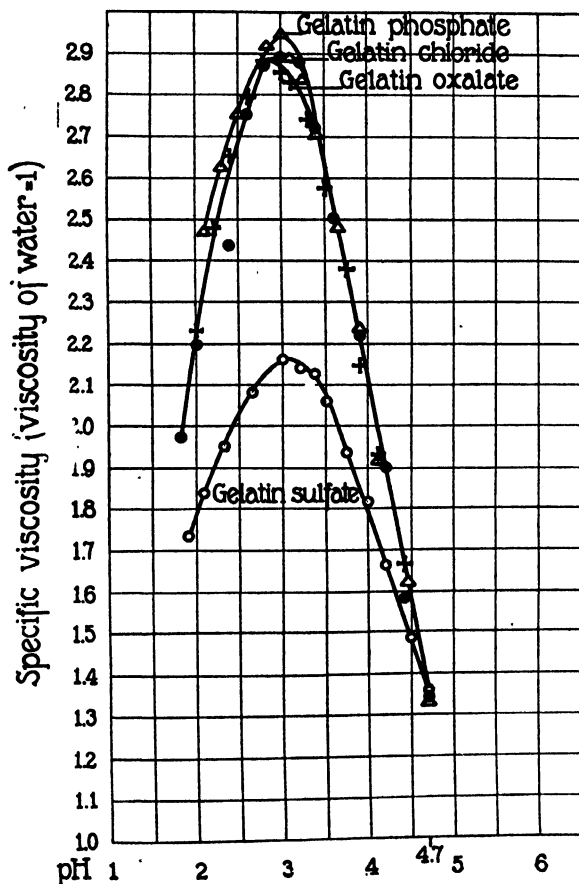
FIG. 10. The curves of specific viscosity of 1 per cent solution of originally iso-electric gelatin brought to different pH by the acids mentioned in legend of Fig. 9 except that HCl is used for HNO₃. The curves of viscosity for gelatin chloride, phosphate, and oxalate are practically identical. Specific viscosity given as time of outflow of gelatin solution divided by time of outflow of water through viscometer at 24°C.

of brevity we will call this quotient the specific viscosity of the gelatin solution.   The curves for the four acids all rise steeply from the iso-electric point with increasing hydrogen ion concentration until they reach a maximum at pH about 3.0.   The curves then drop again.
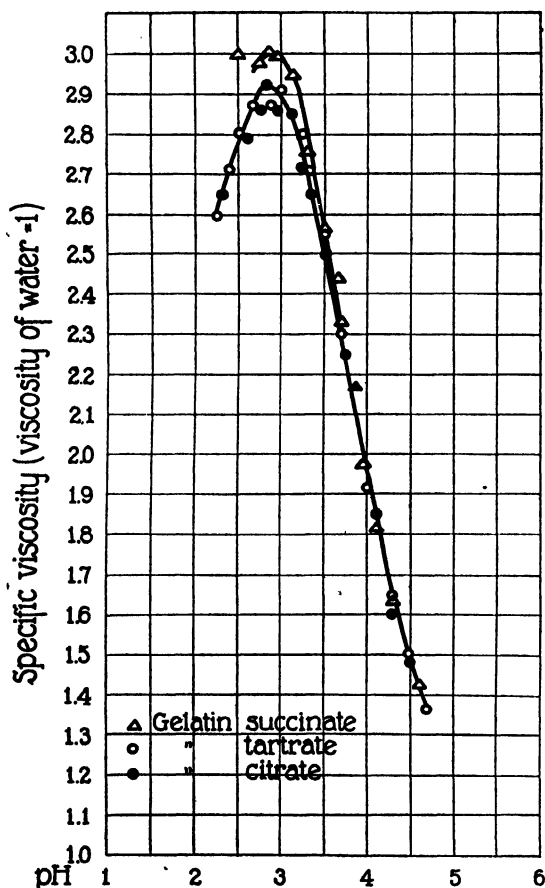
FIG. 11. Curves of specific viscosity of gelatin succinate, tartrate, and citrate. The curves are practically identical with those for the viscosity of gelatin chloride and phosphate.

The curves for the three acids, gelatin chloride, oxalate, and phosphate, are practically identical while the curve for gelatin sulfate is considerably lower.

Fig. 11 gives the curves for the viscosity of gelatin citrate, tartrate, and succinate. The three curves are practically identical and also identical with the curves for gelatin chloride and gelatin phosphate in Fig. 10.
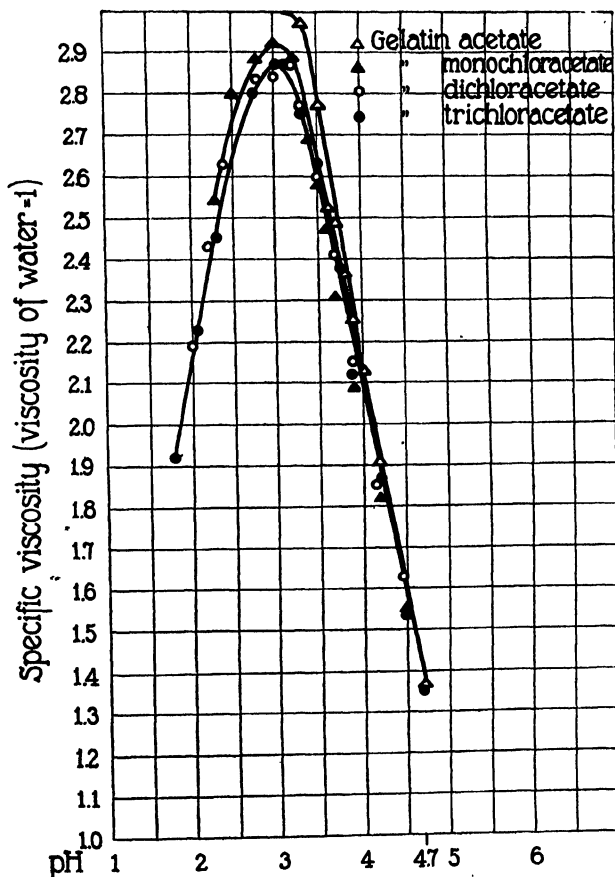
FIG. 12. Curves of specific viscosity of gelatin acetate, mono-, di-, and trichloracetate. Curves identical with those for gelatin chloride and phosphate.

Fig. 12 gives the curves for the viscosity of 1 per cent solutions of originally isoelectric gelatin to which acetic and mono-, di-, and trichloracetic acids have been added. The curves are again identical
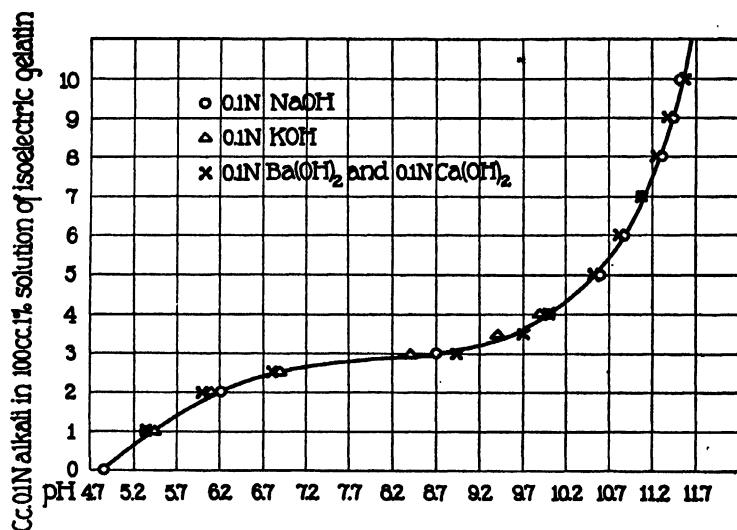
Fig. 13. Curves for the number of cc. of 0.1 N NaOH, KOH, Ba(OH)₂, and Ca(OH)₂ required to bring 1 gm. of isoelectric gelatin to different pH (in 100 cc. of solution). All four curves are identical.
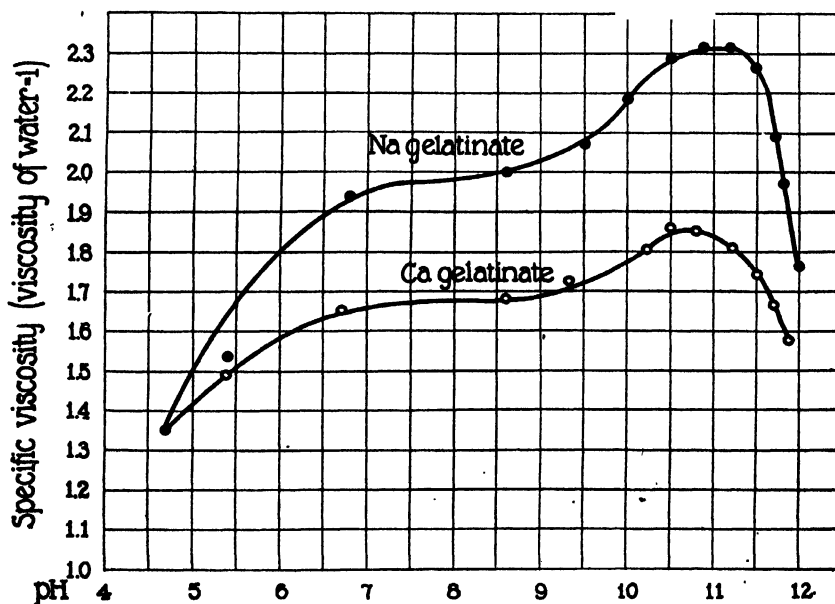
with those for gelatin chloride, phosphate, etc. These curves are interesting in connection with Pauli's statement that· trichloracetic acid causes the same low values of viscosity as sulfuric acid. This was the case neither in our experiments on the osmotic pressure nor in those on the viscosity of gelatin solutions.

Fig. 13 gives the cc. of 0.1 N NaOH, KOH, Ca(OH)$_2$, and Ba(OH)$_2$ required to bring 1 gm. of originally isoelectric gelatin in 100 cc. solution to a given pH. One curve suffices for the four alkalies thus proving that Ca and Ba combine with gelatin as bivalent ions. We should expect the curve for the viscosity of Ca gelatinate to be considerably lower than that of Na gelatinate. Fig. 14 shows that this is true.

SUMMARY.

1. This paper contains experiments on the influence of acids and alkalies on the osmotic pressure of solutions of crystalline egg albumin and of gelatin, and on the viscosity of solutions of gelatin.

2. It was found in all cases that there is no difference in the effects of HCl, HBr, HNO$_3$, acetic, mono-, di-, and trichloracetic, succinic, tartaric, citric, and phosphoric acids upon these physical properties when the solutions of the protein with these different acids have the same pH and the same concentration of originally isoelectric protein.

3. It was possible to show that in all the protein-acid salts named the anion in combination with the protein is monovalent.

4. The strong dibasic acid H$_2$SO$_4$ forms protein-acid salts with a divalent anion SO$_4$ and the solutions of protein sulfate have an osmotic pressure and a viscosity of only half or less than that of a protein chloride solution of the same pH and the same concentration of originally isoelectric protein. Oxalic acid behaves essentially like a weak dibasic acid though it seems that a small part of the acid combines with the protein in the form of divalent anions.

5. It was found that the osmotic pressure and viscosity of solutions of Li, Na, K, and NH$_4$ salts of a protein are the same at the same pH and the same concentration of originally isoelectric protein.

6. Ca(OH)$_2$ and Ba(OH)$_2$ form salts with proteins in which the cation is divalent and the osmotic pressure and viscosity of solutions of these two metal proteinates are only one-half or less than half of

that of Na proteinate of the same pH and the same concentration of originally isoelectric gelatin.

7. These results exclude the possibility of expressing the effect of different acids and alkalies on the osmotic pressure of solutions of gelatin and egg albumin and on the viscosity of solutions of gelatin in the form of ion series. The different results of former workers were probably chiefly due to the fact that the effects of acids and alkalies on these proteins were compared for the same quantity of acid and alkali instead of for the same pH.

AN INVESTIGATION OF THE SIZE OF THE HEART IN SOLDIERS BY THE TELEROENTGEN METHOD.

BY ALFRED E. COHN, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research.)

It is not known with sufficient accuracy whether exertion, such as soldiers undergo in warfare, is accompanied by enlargement of the heart. The present study was undertaken to obtain information on this point. The examinations were made during May, 1919, of soldiers who had seen active service in the American Expeditionary Forces.[1]

The men were selected without regard to special criteria by line officers at one of the camps in the vicinity of New York.[2] Infantry-men were chosen or men who had been subjected to an equivalent amount of privation and exertion. In this report are given the results of the study of the size of the heart only. Physical examinations were made and electrocardiograms were taken, but the results of these further studies are reserved for later communications.

The examinations now reported were roentgenographic.[3] The exposures were made with sternum turned to, and parallel with, the plate. The distance from the anticathode of the roentgen-ray tube to the photographic plate was 6 feet. A strip of lead about 10.0 cm. long, 6.0 mm. wide, and about 3.0 mm. thick was laid on the skin over the spines of the vertebrae and secured with adhesive plaster. Two acute angles of lead were similarly secured—one in the suprasternal notch and the other in the infrasternal notch. The target of the

roentgen-ray tube was adjusted to the level of the lower angle. Whether correct anteroposterior alignment was obtained, could then be ascertained by examining the plate.[4]   The exposures were made in the standing position.   In making studies of this kind, Bardeen[5] made the exposure "during deep but not forced inspiration and with two half second exposures with an intervening half second so as to insure a diastolic outline."   The exposures were made by Smith[6] during an inspiration of moderate depth after the subject had taken a deep breath and expired it.   A method involving deeper breathing than normal has this purpose: it frees a larger portion of the cardiac shadow from the shadows of the surrounding viscera, especially the liver, and it permits the drawing of the outline of the cardiac shadow with greater ease.   It is admitted that there is a disadvantage in the procedure; when the breath is held too long it occasions too great a filling of the heart, the photograph of which is, in consequence, larger than normal.   A modification of the usual technic was, therefore, introduced in this study.   No directions for breathing during the exposure were given; the men breathed normally.   In order that the phase of respiration in which the plate was secured might be known, the following technic was devised.

On the plate holder a lead strip was secured to indicate the neutral position of the vertically hanging lever of a Marey tambour tipped with a lead ring.   The tambour was connected by rubber tubing with a Politzer bag held in position in the right axilla by a binder secured by tying its tails; metal fastenings were, of course, avoided.   During respiration, the lever swung to one or other side of the neutral line indicated by the lead strip.   The side to which the lever swung during expiration was indicated on the plate holder by fastening there a lead letter E.   The exposures were brief, a fraction of a second, perhaps as little as one-tenth second on occasion, so that the image of the lead

4. Mention is made of this technical detail because the use of this method secures greater accuracy than that which employs a lead strip on the sternum and a ring over a vertebral spine.

5. Bardeen, C. R.: Determination of the Size of the Heart by Means of the X-Ray, Am. J. Anat. 23: 423, 1918.

6. Smith, B.: Teleroentgen Measurements of the Hearts of Normal Soldiers, Arch. Int. Med., this issue, p. 522.

TABLE 1.

Data and Measurements of the Hearts of the Soldiers Examined.

No.	Phase Respiration	P.	Weight, Kg.	Height, Cm.	Age, Yrs.	Transverse Diameter, Cm.	Long Diameter, Cm.	Area, Sq. Cm.	Angle, Degrees	Notes
1	I		53	157	21	13.4	13.4	94	38	
2	I ?	+	53	152	29	12.7	13.6	102	37	Wounded
3	I		54	164	40	12.0	13.1	108	46	
4	I	+	54	162	20	11.9	13.3	86	40	
5	I		55	158	29	12.8	14.5	122	42	
6	I	+	56	158	25	13.9	13.9	120	43	
7	I		56	173	24	13.8	15.7	112	42	
8	I		56	171	27	12.3	14.3	103	47	
9	I	+	56	168	22	11.2	13.2	92	47	Gas
10	I		57	160	19	12.3	13.8	109	47	Gas
11	I		57	168	24	12.6	15.0	110	47	
12	N		57	172	26	11.5	14.3	110	51	
13	?		57	165	23	11.2	13.7	100	56	Appendicitis
14	?		58	163	24	12.8	13.7	112	37	Wounded
15	I		58	168	25	11.4	13.2	94	52	
16	I		58	170	25	11.2	13.0	94	49	Gas
17	I		59	175	19	12.5	13.9	122	46	Gas
18	I ?		59	169	21	11.5	12.5	99	49	Bronchitis
19	?		59	166	25	11.5	14.0	108	48	
20	I	+	59	167	23	12.5	14.7	108	43	
21	E		60	172	25	12.7	13.6	100	34	Gas, rheumatism? dysentery
22	I		60	174	29	11.3	13.0	98	41	
23	E		60	171	25	12.4	14.2	110	42	
24	I		60	162	23	13.3	14.3	106	36	
25	I		60	164	29	13.3	15.1	109	45	Wounded
26	I		60	183	25	12.5	14.3	101	45	
27	I		60	174	21	10.8	13.0	96	46	
28	?		60	173	23	12.5	13.4	107	45	
29	I		60	170	24	12.5	13.7	104	43	
30	I	+	61	161	27	12.8	13.7	103	39	Wounded
31	I		61	174	24	11.8	12.7	102	35	Wounded, gas
32	I	+	61	168	29	10.5	12.5	92	44	Gas, shell shock
33	E		61	164	25	13.0	13.6	113	41	
34	I	+	61	176	27	11.7	13.8	95	47	Appendicitis

TABLE 1—*Continued.*

No.	Phase Respiration	P.	Weight, Kg.	Height, Cm.	Age, Yrs.	Transverse Diameter, Cm.	Long Diameter, Cm.	Area, Sq. Cm.	Angle, Degrees	Notes
35	I		62	163	24	11.2	13.7	121	48	
36	I		62	166	30	14.2	14.6	124	39	
37	I		62	173	29	11.9	13.9	108	50	Gas
38	I	+	62	171	26	12.3	13.2	93	43	
39	?	+	62	173	25	12.5	14.6	109	44	Gas
40	E		62	170	29	13.0	15.0	105	42	Gas
41	E		63	172	50	12.8	11.8	84	32	Gas
42	I	+	63	163	33	14.0	15.0	109	33	
43	I		63	163	26	13.5	14.2	103	34	Gas, wounded, rheumatism ?
44	I		63	174	40	13.8	14.0	112	42	Gas
45	?		63	· 169	21	13.5	14.9	130	44	
46	I		63	167	24	11.8	13.4	101	45	
47	I		63	164	21	12.8	14.0	127	45	Shell shock
48	I		63	169	28	12.0	14.0	100	51	
49	I		63	178	23	12.7	13.5	109	50	
50	I		63	170	23	11.3	13.5	97	48	
51	I	+	63	172	20	10.5	13.1	91	53	
52	'I		64	167	30	14.4	14.6	125	36	
53	N		64	164	29	12.0	12.5	83	34	
54	E		64	174	23	12.8	13.5	102	38	Gas
55	E		64	167	31	12.7	13.8	104	38	Wounded
56	N		64	173	24	13.4	14.4	129	47	Gas
57	I	+	64	176	21	12.2	13.5	82	38	
58	I	+	64	167	24	12.5	13.9	100	38	Gas
59	I		64	181	29	11.2	13.5	98	45	
60	I		64	171	26	12.8	14.4	108	43	Trench fever ?
61	I	+	64	168	28	13.2	15.2	131	47	Gas
62	I		65	177	28	14.2	14.8	126	38	
63	I	+	65	175	28	13.3	14.6	119	40	
64	I		65	168	20	12.8	13.4	109	42	Gas
65	E		65	170	20	11.8	14.0	114	47	
66	I		65	176	21	12.8	13.3	112	45	Gas, shell shock
67	I	+	65	164	27	12.3	12.8	96	36	Wounded
68	I		65	172	27	12.8	14.0	111	44	
69	I		65	170	23	12.7	15.3	126	50 ·	
70	E		65	170	20	11.2	13.2	95	48	

TABLE 1—*Continued.*

No.	Phase Respiration	P.	Weight, Kg.	Height, Cm.	Age, Yrs.	Transverse Diameter, Cm.	Long Diameter, Cm.	Area, Sq. Cm.	Angle, Degrees	Notes
71	I	+	66	165	22	11.6	13.4	92	39	
72	I		66	164	27	13.5	15.4	126	40	Gas
73	E		66	182	25	12.2	13.6	101	44	
74	I		66	167	26	12.4	14.6	121	53	
75	I	+	66	179	19	12.3	14.5	108	49	
76	E		66	165	21	12.6	15.1	119	48	
77	I		67	161	25	14.5	14.8	109	30	Gas
78	I		67	173	29	14.6	15.3	124	38	Gas
79	I		67	167	25	13.7	15.2	120	38	
80	I	+	67	176	27	13.7	13.5	92	29	Gas
81	I		67	175	23	12.4	13.9	104	37	Gas
82	I		67	167	26	14.4	15.1	109·	35	
83	I		67	175	28	12.4	13.0	95	35	
84	I		67	168	27	14.9	14.6	124	41	
85	I		67	169	23	13.2	13.1	94	32	Gas
86	I		67	170	24	15.0	16.3	137	41	
87	I		68	167	49	13.5	14.0	110	31	
88	E		68	171	24	15.0	15.5	139	39	
89	I		68	171	21	13.4	14.0	111	32	Gas
90	I		68	175	24	12.8	14.1	131	43	
91	I		68	174	18	11.8	12.8	99	36	Gas
92	?		68	170	26	13.8	15.1	118	41	
93	I	+	68	174	20	12.8	14.0	114	47	Gas
94	I		68	175	26	14.0	15.1	118	43	
95	?		68	176	23	12.1	15.4	140	55	Gas
96	I	+	68	178	23	13.0	15.5	140	49	Not in France
97	I		69	175	42	15.7	16.1	132	35	
98	N	+	69	180	26	12.7	13.8	108	35	
99	I	+	69	167	22	12.9	13.5	96	38	Gas
100	I		69	169	26	14.8	15.9	129	37	
101	I		69	174	31	13.8	15.1	122	40	Gas
102	E		69	177	23	12.6	14.1	120	41	
103	I		69	177	25	11.8	13.3	110	48	Gas
104	I	+	69	182	28	12.8	15.0	132	45	Gas, wounded
105	I	+	69	171	30	14.8	15.0	135	41	Gas, wounded
106	I		69	172	26	13.6	15.1	110	42	Gas
107	I	+	69	177	28	12.2	14.3	104	42	
108	I		69	180	28	12.3	13.2	106	49	

TABLE 1—*Continued.*

No.	Phase Respiration	P.	Weight, Kg.	Height, Cm.	Age, Yrs.	Transverse Diameter, Cm.	Long Diameter, Cm.	Area, Sq. Cm.	Angle, Degrees	Notes
109	?		70	174	31	13.5	14.6	112	37	Gas
110	I	+	70	180	30	12.0	13.6	100	40	
111	I		70	171	30	12.8	13.2	95	35	
112	?		70	176	40	15.0	15.0	121	42	
113	I		70	176	29	13.3	14.3	109	36	
114	?		70	170	25	13.9	14.3	120	44	
115	I	+	70	175	24	13.0	14.6	114	40	
116	I ?	+	70	184	27	12.8	14.1	109	46	
117	I		70	175	22	11.8	15.0	109	51	
118	I		70	175	18	13.0	14.0	98	42	
119	I		71	172	22	13.7	15.8	122	35	
120	E		71	167	26	15.0	16.3	136	35	
121	?		71	169	36	14.0	13.8	121	37	
122	I		71	170	29	14.2	14.4	105	32	Gas
123	I		71	177	34	13.3	15.3	110	42	Dysentery ?
124	I	+	71	172	27	14.0	15.7	122	40	Measles
125	I .		71	178	23	12.1	14.0	91	45	
126	I		71	172	23	11.3	13.3	91	43	
127	I		72	179	24	12.7	12.4	94	30	
128	I	+	72	167	24	14.2	14.6	110	37	
129	I		72	174	22	14.0	14.8	134	43	Wounded, buried
130	N		72	165	23	12.4	13.6	104	39	
131	I	+	72	178	24	13.0	15.2	124	45	
132	I		72	174	20	12.4	15.1	122	48	
133	I		72	174	29	14.7	15.7	127	42	
134	?		72	177	26	13.4	14.5	123	45	
135	I		72	173	21	12.8	15.3	127	52	
136	I	+	73	173	25	14.5	14.9	110	34	
137	I		73	178	21	14.1	14.8	119	38	
138	I		74	174	28	14.4	15.0	114	35	Gas
139	I	+	74	166	22	14.6	14.1	113	33	
140	I		74	171	24	13.7	14.7	110	34	
141	I		74	172	23	12.3	12.7	80	33	
142	I		74	172	25	13.0	14.3	115	45	
143	I		74	178	27	13.3	16.1	146	46,	
144	I		74	186	23	12.7	14.3	96	41	

TABLE 1—*Concluded*.

No.	Phase Respiration	P.	Weight, Kg.	Height, Cm.	Age, Yrs.	Transverse Diameter, Cm.	Long Diameter, Cm.	Area, Sq. Cm.	Angle, Degrees	Notes
145	I	+	75	175	25	14.7	15.8	115	33	
146	I	+	75	170	28	13.2	14.4	95	37	
147	I		75	173	25	16.0	16.2	108	21	
148	I	+	75	172	25	12.0	13.2	100	39	
149	I		75	165	27	13.0	14.2	118	45	
150	I		75	175	30	13.2	15.3	114	41	
151	?		75	174	21	12.7	14.4	102	45	
152	N		75	175	24	11.0	14.1	111	56	
153	I	+	76	176	25	14.5	15.3	121	33	Febricula
154	I		77	179	28	12.3	15.0	127	48	
155	I		77	181	30	12.8	15.9	126	49	Trench fever ?
156	E		78	173	24	14.2	14.3	110	28	Gas
157	I		78	170	28	14.3	14.8	107	35	
158	I		83	180	27	14.3	15.8	120	39	
159	I	+	83	182	21	12.5	14.7	124	44	Wounded
160	I		83	179	26	13.8	16.6	127	46	
161	I		86	180	26	13.8	14.7	110	36	

Column 2 gives the phase of respiration in which the exposure is made.

Column 3 indicates (+) whether exposures were obtained both in inspiration and expiration.

Columns 4, 5, and 6 give the weights, height, and age.

· Columns 7, 8, 9 and 10 give the transverse diameter, the long diameter, the area of the cardiac shadow, and the cardiac angle.

Column 11 gives notes of interest.

ring of the lever was sharp. When the exposure was longer, or the breathing faster, the trail of the lever, as it swung across the plate, appeared on the plate and showed this fact. The lever of the tambour was in view of the operator, so that by observing it the exposure could be made in any phase of breathing. The desired information was, accordingly, recorded on the plate. The attempt was made to secure the exposures during normal inspiration. This succeeded in 140 of 161 instances (I, ?, Table 1).

But to study the effect of normal breathing on the size of the heart, the heart in fifty-six instances was photographed both in inspiration and in expiration. Note should be taken of the fact that in many persons expiration is not an active process, but is the release from

TABLE 2.

Averages of Table 1.

No. of Cases	Weight, Kg.	Height, Cm.	Age, Yrs.	Transverse Diameter, Cm.	Long Diameter, Cm.	Area, Sq. Cm.	Angle, Degrees
2	53	154	25	13.0	13.5	98	37
2	54	163	30	11.9	13.2	97	43
1	55	158	29	12.8	14.5	122	42
4	56	167	24	12.8	14.2	106	44
4	57	166	23	11.9	14.2	107	50
3	58	167	24	11.8	13.3	100	46
4	59	169	22	12.0	13.7	111	46
9	60	171	24	12.3	13.8	103	41
5	61	168	26	11.9	13.2	101	41
6	62	169	27	12.5	14.1	110	44
11	63	169	28	12.6	13.8	105	43
10	64	170	26	12.7	13.9	106	40
9	65	171	23	12.6	13.9	112	43
6	66	170	23	12.4	14.4	111	45
10	67	170	25	13.7	14.4	110	35
10	68	173	25	13.2	14.5	122	41
12	69	175	28	13.3	14.5	117	41
10	70	175	27	13.1	14.2	108	41
8	71	172	27	13.4	14.8	112	38
9	72	173	23	13.2	14.5	118	43
2	73	175	23	14.3	14.8	114	36
7	74	174	24	13.4	14.4	110	38
8	75	172	25	13.2	14.7	107	39
1	76	176	25	14.5	15.3	121	33
2	77	180	29	12.5	15.4	126	48
2	78	171	26	14.2	14.5	108	31
3	83	180	24	13.5	15.7	123	43
1	86	180	26	13.8	14.7	110	36

inspiration. In these cases, the expiratory phase is represented by the neutral position (N, Table 1). A question mark in the table indicates that the ring was not identified in the plate; it swung beyond the plate. There is, however, a defect in this method. It is impossible to record on the plate the exact level of the respiratory phase at which

the exposure was made. The method is subjective to the extent that to secure the photograph at the height of the respiratory phases, reliance is placed on the operator.

The measurements taken were those recommended by Moritz[7] and his followers. In the plates the right and left borders were traced, and the outline of the heart's shadow completed by joining arbitrarily the lines representing these. The long and transverse diameters were measured. The angle of inclination of the heart, that is to say, the angle formed by the long diameter and a line drawn to the cardiac apex at right angles with the median line, as recorded. The area of the cardiac outline was measured by the planimeter.

In measuring fifty-six pairs of roentgenograms (Tables 3 and 4) it was found that in inspiration and in expiration the transverse diameter was identical in six pairs, greater in inspiration in thirty-one pairs and smaller in nineteen pairs. The long diameters were identical in three pairs, greater in inspiration in forty-two pairs, and smaller in eleven pairs. The area was greater in inspiration in forty-four pairs, smaller in twelve pairs. The angle of inclination was identical in three pairs, greater in inspiration in thirty-nine pairs, and smaller in fourteen pairs. But the difference in size and the difference in position were not great. The transverse diameters (less subject to error, as will be pointed out later, than the long diameter, because it does not require the arbitrary location of the apex) differed 5 mm. or less in forty-two pairs, and 1.0 cm. or less in fifty-two pairs. The long diameters differed 5 mm. or less in thirty pairs, and 1.0 cm. or less in forty-eight pairs. The areas differed by 5.0 sq. cm. or less in twenty-two pairs, and by 10.0 sq. cm. in thirty-seven pairs. Significant differences in size, therefore, do not usually occur during the inspiration and expiration of normal breathing. But the fact that arrests attention is that the heart shadows are larger, that they have longer diameters and that during inspiration the angles increase. There

7. Moritz, F.: Ueber Veränderungen in der Form, grösse und Lage des Herzens beim Uebergang aus horizontalen in vertikale Körperstellung. Zugleich ein zweiter Beitrag zur Methodik der Orthodiagraphie, insbesonders zu der Frage wie die Orthodiagramme auszumessen seien und welche Körperstellung für die Orthodiagraphie des Herzens zu wählen sei, Deutsch. Arch. f. klin. Med. 82: 1, 1905.

TABLE 3.

Measurements of the Cardiac Shadow in Inspiration and Expiration, and the Difference between the Two.

No.	Inspiration				Expiration				Difference Between Inspiration and Expiration			
	Area, Sq. Cm.	Long Diameter, Cm.	Transverse Diameter, Cm.	Angle, Degrees	Area, Sq. Cm.	Long Diameter, Cm.	Transverse Diameter, Cm.	Angle, Degrees	Area, Sq. Cm.	Long Diameter, Cm.	Transverse Diameter, Cm.	Angle, Degrees
1	106	14.1	13.3	32	116	14.4	13.3	35	−10	−0.3	0.0	−3
2	101	15.0	13.7	33	99	14.5	14.4	29	+ 2	+0.5	−0.3	+4
3	88	12.2	12.7	33	78	11.9	12.5	31	+10	+0.3	+0.2	+2
4	92	13.5	13.7	29	83	12.7	12.8	29	+ 9	+0.8	+0.9	0
5	95	13.0	13.0	35	85	12.5	12.7	34	+10	+0.5	+0.3	+1
6	109	15.0	14.4	33	102	14.6	14.0	32	+ 7	+0.4	+0.4	+1
7	115	15.8	14.7	33	112	14.9	14.3	33	+ 3	+0.9	+0.4	0
8	121	15.3	14.5	33	103	13.4	14.0	27	+18	+1.9	+0.5	+6
9	113	14.1	14.6	33	117	14.7	14.7	33	− 4	−0.6	−0.1	0
10	100	13.6	12.0	40	97	13.1	12.5	39	+ 3	+0.5	−0.5	+1
11	110	14.6	14.2	37	95	13.4	12.7	38	+15	+1.2	+1.5	−1
12	90	12.9	12.2	36	94	13.0	12.4	34	− 4	−0.1	−0.2	+2
13	114	13.9	12.8	34	96	12.8	11.6	39	+18	+1.1	+1.2	−5
14	108	13.8	12.7	35	102	13.7	12.6	36	+ 6	+0.1	+0.1	−1
15	109	14.7	12.5	40	104	13.9	13.2	36	+ 5	+0.8	−0.7	+4
16	95	14.4	13.2	37	98	14.4	13.6	34	− 3	0.0	−0.4	+3
17	110	14.9	14.5	34	107	14.9	14.3	35	+ 3	0.0	+0.2	−1
18	113	14.5	14.0	41	104	13.9	13.4	35	+ 9	+0.6	+0.6	+6
19	103	13.7	12.8	39	100	13.9	13.0	38	+ 3	−0.2	−0.2	+1
20	92	13.4	11.6	39	96	12.8	11.3	40	− 4	+0.6	+0.3	−1
21	95	13.3	12.0	43	93	13.0	11.7	39	+ 2	+0.3	+0.3	+4
22	96	13.5	12.9	38	98	13.4	13.2	35	− 2	+0.1	−0.3	+3
23	122	16.2	14.0	39	108	14.6	13.5	34	+14	+1.6	+0.5	+5
24	100	13.2	12.0	39	103	13.5	12.9	35	− 3	−0.3	−0.9	+4
25	109	14.5	13.8	41	104	13.7	13.5	34	+ 5	+0.8	+0.3	−7
26	132	14.8	13.7	42	112	14.3	13.5	36	+20	+0.5	+0.2	+6
27	128	14.8	13.3	44	124	14.5	13.0	45	+ 4	+0.3	+0.3	−6
28	82	13.5	12.2	38	85	13.0	12.2	39	− 3	+0.5	0.0	−1
29	119	14.6	13.3	40	95	12.4	12.8	42	+24	+2.2	+0.5	−2
30	122	15.7	13.4	41	108	14.1	13.4	38	+14	+1.6	0.0	+3
31	100	13.9	12.5	38	91	13.6	12.6	37	+ 9	+0.3	−0.1	+1
32	92	12.5	10.5	44	80	12.3	10.3	37	+12	+0.2	+0.2	+7
33	120	13.9	13.9	43	107	13.5	12.8	40	+13	+0.4	+1.1	+3
34	114	14.0	12.8	47	102	13.0	12.0	36	+12	+1.4	+0.8	+11
35	98	13.8	12.4	42	104	14.4	13.3	38	− 6	−0.6	−0.9	+4
36	122	15.7	14.0	40	110	15.8	13.9	39	+12	−0.1	+0.1	+1
37	114	14.6	13.0	40	105	14.0	13.0	42	+ 9	+0.6	0.0	−2
38	132	15.0	12.8	45	115	14.5	12.7	41	+17	+0.5	+0.1	+4
39	95	13.8	11.7	47	90	13.1	11.7	41	+ 5	+0.7	0.0	+6

TABLE 3—*Concluded.*

No.	Inspiration				Expiration				Difference Between Inspiration and Expiration			
	Area, Sq. Cm.	Long Diameter, Cm.	Transverse Diameter, Cm.	Angle, Degrees	Area, Sq. Cm.	Long Diameter, Cm.	Transverse Diameter, Cm.	Angle, Degrees	Area, Sq. Cm.	Long Diameter, Cm.	Transverse Diameter, Cm.	Angle, Degrees
40	124	14.7	12.5	44	104	13.9	13.6	37	+20	+0.8	−1.1	+7
41	140	15.5	13.5	49	124	14.7	13.0	40	+16	+0.8	+0.5	+9
42	124	15.2	13.0	45	103	13.8	12.4	38	+21	+1.4	+0.6	+7
43	86	13.3	11.4	40	82	13.1	11.9	35	+ 4	+0.2	−0.5	+5
44	109	14.1	12.8	46	102	14.1	12.2	41	+ 7	0.0	+0.6	+5
45	108	14.5	12.3	49	96	13.6	12.8	38	+12	+0.9	−0.5	+1
46	109	13.8	12.8	42	108	14.0	13.0	41	+ 1	−0.2	−0.2	+1
47	93	13.2	12.3	43	80	12.6	12.2	37	+13	+0.6	+0.1	+6
48	92	13.2	12.2	47	100	13.5	11.5	50	− 8	−0.3	+0.7	−3
49	104	14.3	12.2	42	94	14.0	12.3	35	+10	+0.3	−0.1	+7
50	109	14.6	12.5	44	98	13.1	12.2	41	+11	+1.5	+0.3	+3
51	108	14.7	12.5	43	110	14.9	13.2	41	− 2	−0.2	−0.7	+2
52	91	13.1	10.5	53	87	12.5	10.6	54	+ 4	+0.6	−0.1	−1
53	131	15.2	13.2	47	121	14.4	12.8	48	+10	+0.8	+0.4	−1
54	102	13.6	12.5	37	105	13.8	12.7	34	− 3	−0.2	−0.2	+3
55	135	15.0	14.8	41	117	14.1	14.3	33	+18	−0.9	+0.5	+8
56	96	12.8	12.3	36	87	12.7	12.3	32	+ 9	+0.1	0.0	+4

+ indicates that inspiration is greater than expiration.

− indicates that inspiration is smaller than expiration.

appears no reason to doubt that in certain individuals decrease in these measurements takes place, but the study of the roentgenograms supplies no explanation for this occurrence. The reason may be sought, perhaps, in peculiarities in the motion of the thorax and of the diaphragm.

The comparison of roentgenograms made in normal inspiration and expiration then, permits the statement that the difference between the two is not great; that, as is expected, the size of the heart shadow is usually greater in inspiration; and that, in this phase, the angle of inclination is usually larger. For clinical purposes, therefore, as will be shown, in a method accompanied by variations so large, the influence of the phases of normal respiration on the size of the heart may be neglected.

HEART MEASUREMENTS

TABLE 4

Differences in the Measurements Tabulated between Inspiration and Expiration.

Area		Long Diameter		Transverse Diameter		Angle	
Sq. Cm.	No. of cases	Cm.	No. of Cases	Cm.	No. of Cases	Degrees	Cases
+24	1						
+22		+2.2	1				
+21	1	+2.1					
+20	2	+2.0					
+19		+1.9	1				
+18	3	+1.8					
+17	1	+1.7					
+16	1	+1.6	2				
+15	1	+1.5	1	+1.5	1		
+14	2	+1.4	1	+1.4			
+13	2	+1.3		+1.3			
+12	4	+1.2	1	+1.2	1		
+11	1	+1.1	1	+1.1	1	+11	2
+10	4	+1.0	1	+1.0		+10	
+ 9	5	+0.9	3	+0.9	1	+ 9	1
+ 8		+0.8	6	+0.8	1	+ 8	1
+ 7	2	+0.7	1	+0.7	1	+ 7	4
+ 6	1	+0.6	5	+0.6	3	+ 6	4
+ 5	3	+0.5	6	+0.5	5	+ 5	3
+ 4	3	+0.4	2	+0.4	3	+ 4	7
+ 3	4	+0.3	5	+0.3	6	+ 3	6
+ 2	2	+0.2	2	+0.2	4	+ 2	3
+ 1	1	+0.1	3	+0.1	4	+ 1	8
0		0	3	0	6	0	3
− 1		−0.1	2	−0.1	4	− 1	7
− 2	2	−0.2	4	−0.2	4	− 2	2
− 3	4	−0.3	3	−0.3	2	− 3	2
− 4	3	−0.4		−0.4	1	− 4	
− 5		−0.5		−0.5	3	− 5	1
− 6	1	−0.6	2	−0.6		− 6	1
− 7		−0.7		−0.7	2	− 7	1
− 8	1	−0.8		−0.8			
− 9				−0.9	2		
−10	1			−1.3	1		

Grouped totals (braces): Area — 44, 22, 37, 12. Long Diameter — 42, 30, 48, 3, 11. Transverse Diameter — 31, 42, 52, 6, 19. Angle — 39, 3, 14.

\+ indicates that inspiration is larger than expiration.

− indicates that inspiration is smaller than expiration.

The hearts were measured in 208 soldiers. Although the men who were sent were considered by themselves and by the officers who selected them sound and fit for service, forty-seven had, at one time or another, suffered from an acute infectious disease. The infections and the number of men affected were as shown in Table 5. Only the remaining 161 are considered, therefore, in this discussion (Table 1). For the sake of completeness and for the use of others making similar studies the data of the forty-seven other men are given (Tables 6 and 7).

TABLE 5.

Infectious Diseases Reported by the Rejected Soldiers.

Disease	No. of Cases	Other Diseases	No. of Cases	Disease	No. of Cases	Other Diseases	No. of Cases
Typhoid fever...	2			Dysentery.....	2;	also Pneumonia....	1
Trench fever....	2					Influenza......	1
Influenza.......	11;	also Trench fever	1			Trench fever	
Gas poisoning...	6;	also Dysentery...	2			and gas.....	1
		Pneumonia..	2	Mumps.......	5;	also Gas poisoning.	1
		Rheumatism	1	Pneumonia....	8		
				Scarlet fever...	1		

Table 1 gives the following information: The phase of respiration in which each plate was secured; whether plates of both respiratory phases were taken; the weight, height and age; the transverse diameter, the long diameter, and area of the cardiac shadow, and the angle of inclination. Other measurements were not made; they were not considered of special value in this study.

In order to facilitate the study of the data, these are presented in the form of curves. Based on a study of his own[5] and on a collation of published figures, Bardeen[8] has constructed formulae from which he has drawn certain standard curves. In these he has shown that the transverse diameter and the area of the cardiac shadow vary with the weight of the body. For purposes of comparison, these so-called standards are utilized in the following discussion.

The transverse diameter of the heart is considered first. In Bardeen's curve this diameter at 53 kg. is 12.0 cm. (Fig. 1). From this

8. Bardeen, C. R.: Tables for Aid in the Determination of the Relative Size of the Heart by Means of the Roentgen Ray, Am. J. Roentgenol. **4**: 604, 1917.

TABLE 6.

Data and Measurements of the Hearts of the Soldiers Rejected.

No.	Phase Respiration	P.	Weight, Kg.	Height, Cm.	Age, Yrs.	Transverse Diameter, Cm.	Long Diameter, Cm.	Area, Sq. Cm.	Angle, Degrees	Notes
1	I		54	167	25	11.5	12.9	103	50	Typhoid fever
2	I	+	57	172	20	12.0	13.3	95	43	Influenza; trench fever
3	?		58	165	25	13.3	14.7	132	40	Influenza
4	I	+	58	164	25	13.3	14.8	128	44	Gas
5	I		58	161	20	12.4	13.0	96	43	Pneumonia
6	I		58	172	21	11.5	13.6	105	54	Gas
7	I		59	172	24	10.3	13.8	115	59	Influenza
8	I	+	60	167	26	12.4	13.8	98	42	Gas; dysentery
9	I	+	60	163	22	12.8	13.8	109	42	Rheumatism; gas
10	I		61	176	26	12.9	14.2	97	39	Dysentery
11	I		61	168	21	11.2	13.1	88	44	Mumps; gas
12	I		61	173	22	12.3	14.5	128	48	Gas; dysentery
13	I		62	165	25	13.7	15.4	134	40	Mumps
14	I		62	161	29	12.5	14.2	110	42	Gas
15	I	+	63	162	19	12.7	12.2	88	33	Typhoid
16	E		63	171	23	11.5	12.6	91	43	Mumps
17	I		63	174	26	12.0	13.8	118	52	Gas
18	I		63	174	23	11.0	12.7	94	44	Dysentery; pneumonia
19	I		63	175	24	12.9	15.4	131	55	Gas; pneumonia
20	I	+	64	162	22	13.8	14.5	109	41	Mumps
21	I	+	65	169	30	14.0	14.5	113	41	Mumps—kidneys ?
22	I	+	65	171	22	13.7	14.8	132	42	Dysentery; gas; trench fever
23	N	+	66	178	29	12.5	14.7	109	40	Influenza
24	I		66	176	25	12.4	15.7	131	57	Dysentery; influenza
25	I	+	67	168	34	13.3	14.1	106	32	Influenza
26	I		67	172	24	11.8	13.6	110	47	Pneumonia
27	I		67	173	23	12.8	15.1	124	47	Dysentery
28	E		67	172	33	13.0	13.9	104	40	Influenza
29	I		67	179	21	12.8	14.7	119	44	Influenza

TABLE 6—*Concluded.*

No.	Phase Respiration	P.	Weight, Kg.	Height, Cm.	Age, Yrs.	Transverse Diameter, Cm.	Long Diameter, Cm.	Area, Sq. Cm.	Angle, Degrees	Notes
30	N		68	167	31	13.9	14.4	102	35	Gas; pneumonia
31	I	+	69	165	32	13.0	13.0	95	35	Influenza
32	I	+	69	173	26	13.4	15.7	122	41	Trench fever
33	I		70	173	19	14.7	15.2	125	42	Gas
34	I		70	175	22	14.0	14.6	122	44	Trench fever
35	I		71	173	23	13.8	15.2	103	38	Influenza
36	I		72	178	26	15.5	16.4	136	37	Influenza
37	I		72	169	23	11.8	13.6	115	51	Gas
38	I	+	73	173	23	13.7	15.0	101	33	Scarlet fever
39	I		74	176	22	13.8	14.8	133	34	Influenza
40	I		74	163	31	13.5	14.6	113	33	Influenza
41	I	+	74	176	26	14.0	16.2	122	39	Pneumonia
42	I		75	172	29	14.2	14.3	113	36	Pneumonia
43	I		75	163	28	14.4	15.8	125	52	Pneumonia
44	I ?	+	76	176	26	12.2	12.9	90	36	Pneumonia
45	I		80	183	23	14.3	14.5	103	35	Pneumonia
46	I		82	171	32	13.7	14.5	120	42	Pneumonia
47	N	+	86	176	25	12.8	13.9	114	34	Mumps

Column 2 gives the phase of respiration in which the exposure is made.

Column 3 indicates (+) whether exposures were obtained both in inspiration and expiration.

Columns 4, 5, 6 give the weight, height, and age.

Columns 7, 8, 9 and 10 give the transverse diameter, the long diameter, the area of the cardiac shadow, and the cardiac angle.

Column 11 gives notes of interest.

point, the curve follows practically a straight line. The curve obtained in this study from the average measurements of soldiers, follows this with reasonable closeness. Between 56 and 66 kg. and at 74, 75, 77, 83 and 86 kg. the averages are below the standard.

Elsewhere, they equal or exceed it. The differences are not sufficiently great to justify a conclusion that in respect to this dimension the hearts of the soldiers examined differ materially from the normal. The result of the examinations made by Smith[6] parallel these observations closely. Those made by Meakins and Gunson[9] are, on the

TABLE 7.

Averages of Table 6.

No. of Cases	Weight, Kg.	Height, Cm.	Age, Yrs.	Transverse Diameter, Cm.	Long Diameter, Cm.	Area, Sq. Cm.	Angle, Degrees
1	54	167	25	11.5	12.9	103	50
1	57	172	20	12.0	13.3	95	43
4	58	165	22	12.6	14.0	115	45
1	59	172	24	10.3	13.8	115	59
2	60	165	24	12.6	13.8	103	42
3	61	172	23	12.1	13.9	104	40
2	62	163	27	13.1	14.8	124	41
5	63	171	23	12.0	13.3	104	45
1	64	162	22	13.8	14.5	109	41
2	65	170	26	13.8	14.6	122	41
2	66	177	27	12.4	15.2	120	48
5	67	172	27	12.7	14.2	112	42
1	68	167	31	13.9	14.4	102	35
2	69	169	29	13.2	14.3	108	38
2	70	174	20	14.3	14.9	123	43
1	71	173	23	13.8	15.2	103	38
2	72	173	24	13.6	15.0	125	44
1	73	173	23	13.7	15.0	101	33
3	74	171	26	13.7	15.2	122	35
2	75	167	28	14.3	15.0	119	44
1	76	176	26	12.2	12.9	90	36
1	80	183	23	14.3	14.5	103	35
1	82	171	32	13.7	14.5	120	42
1	86	176	25	12.8	13.9	114	34

whole, smaller than the foregoing, but their measurements were taken of the hearts of soldiers who suffered from the "Irritable Heart." Dietlen's[10] figures (Fig. 2), on the other hand, both for soldiers and

9. Meakins, J. C., and Gunson, E. B.: Orthodiagraphic Observations on the Size of the Heart in Cases of So-Called "Irritable Heart," Heart 7: 1, 1918.

10. Dietlen, H.: Ueber Grösse und Lage des normalen Herzens und ihre Abhängigkeit von physiologischen Bedingungen, Deutsch. Arch. f. klin. Med. 88: 55, 1906.

civilians, are a little larger, those for soldiers falling at every weight above the standard, those for civilians falling below it five times. Schieffer's[11] curve (Fig. 2), based on the examination of soldiers in Moritz's clinic, is, on the whole, farther still above the standard; once it coincides with it, and twice it falls below it.

Inasmuch as only the transverse diameter may be measured in an objective manner, being alone uninfluenced by the shadows of other
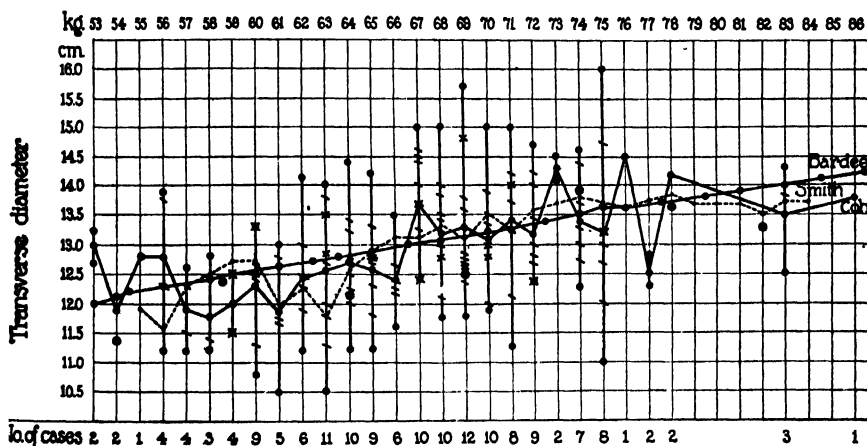
FIG. 1.—The Transverse Diameter.—The ordinates give the diameter; the abscissae the body weight. Below the curves is stated at each weight the number of cases examined at that weight, on which the average is based. The vertical lines represent the range of observations at that weight; the short oblique or horizontal lines which cross these indicate the measurement of each case examined. For comparison the standard curve of Bardeen is given. Smith's curve is likewise shown. ✕ indicates Meakins' and Gunson's average measurement of the hearts of "irritable soldiers."

viscera, especial importance is attached to this measurement. That divergencies appear in the several curves presented is not of so much interest as that the similarity is significant. Although in each series of examinations (Dietlen, Schieffer, Meakins and Gunson, Smith, Cohn) the number at each weight examined was not great, the curves

11. Schieffer: Ueber den Einfluss des Militärdienstes auf die Herzgrösse, Deutsch. Arch. f. klin. Med. 92: 392, 1908.

show that at succeeding weights a gradual, even if not uniform, increase in length of this diameter occurs. Since these curves are based on averages, the range of the individual measurements which form the bases of them requires consideration. Bardeen's standard curve, it will be remembered, is constructed from a formula. The widest ranges observed in the present study were 3.5 cm. at 63 kg. (11 cases); 3.8 cm. at 69 kg. (12 cases) and 5.0 cm. at 75 kg. (8 cases). But at 63 kg., 8 of 11 cases fell within 2.0 cm.; at 69 kg., 7 of 12 cases
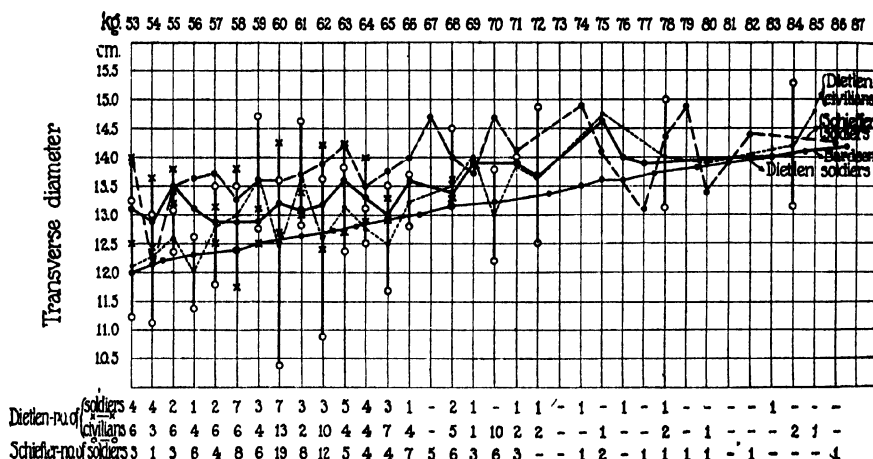
FIG. 2.—The Transverse Diameter.—The ordinates give the diameter; the abscissae the body weight. The dotted line represents Dietlen's civilians. The heavy broken line represents Schieffer's soldiers. For comparison the light unbroken line representing the standard curve of Bardeen is given; and the heavy line represents Dietlen's civilians. The vertical lines at each weight represent the range of Dietlen's observations; x———————x, the range for soldiers; o———————o, the range for civilians. Below the curve at each weight is stated the number of observations at that weight, on which the average is based.

fell within 1.2 cm.; at 75 kg., 5 of 8 cases fell within 1.2 cm. The ranges which are usual are, therefore, not wide. In Dietlen's fifty-nine soldiers these were, for example: at 53 kg., 1.5 cm.; at 54 kg., 1.2 cm.; at 58 kg., 2.1 cm.; at 60 kg., 1.6 cm.; at 62 kg., 1.8 cm.; at 63 kg., 1.5 cm.; at 65 kg., 0.4 cm. If the civilians are included they were: at 53 kg., 2.8 cm.; at 54 kg., 2.5 cm.; at 58 kg., 2.1 cm.; at 60 kg., 3.9 cm.; at 62 kg., 3.3 cm.; at 63 kg., 1.8 cm.; at 65 kg., 1.8 cm.

The curve of the average long diameter is compared with the curve obtained by Smith (Fig. 3). The two approximate each other closely. The difficulty of locating the apex precisely in the photographic plate makes the estimation of the long diameter inexact. The same difficulty is experienced in outlining and measuring the area of the heart's shadow. It is, therefore, considered under that head.

To delimit the area of the cardiac shadow requires that the extremities of the lines representing the right and left borders of the heart be joined by curved lines drawn arbitrarily. With experience these lines are probably drawn by different observers in a consistent manner, but
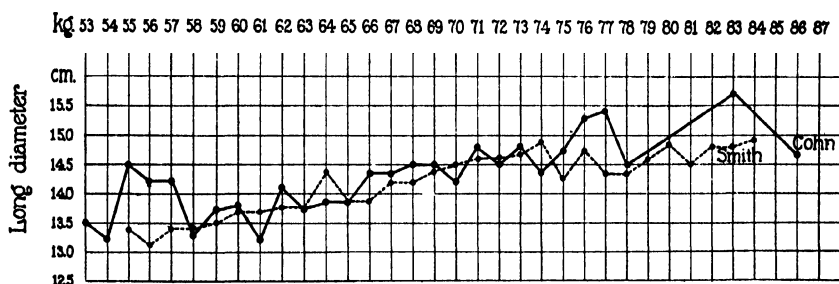
FIG. 3.—The Long Diameter.—The ordinates give the diameter; the abscissae the body weight. The unbroken line represents the curve of the present observations. Those of Smith are given for comparison. The number of men examined at each weight is the same as in Curve 1.

no doubt each observer draws them differently. The outline, as Bardeen[5] points out, "will include within the territory of the heart the right and left atria and the cardiac extremity of the pulmonary artery and of the aorta. A small portion of the left auricle may be cut off by the line that curves toward the right from the left border, but as a rule this is insignificant." The outline is, therefore, as Bardeen says, a modified outline of the heart. But the completed outline fails in exact representation also because of the difficulty, indeed, often because of the impossibility, of locating the apex in certain individuals. On being able to locate this point accurately depends the value of the measurement, both of the long diameter and of the area. Although the subject of heart disease is not considered in this paper, it may be pointed out that this difficulty is increased further in cases of heart

failure, because the maneuver of exposing by deep breathing a length of the left border of the heart shadow sufficiently great to insure accuracy is often extremely difficult. Mention is made here of heart failure because a technic for the study of the size of the heart in this condition and in the stages which lead to it is a matter of concern in the clinic, where criteria for judging of size are earnestly wanted. For this reason, and in order to avoid the exaggeration in size due to deep breathing, the method of breathing while securing the roentgenograms was modified. In addition to the difficulty of locating the apex, the method of completing the outline from the apex to the right border also varies, as has been said, with individual observers. In this study, for instance, the lower outline was drawn in a conservative manner so that the cardiac area was less by several square centimeters than that which might have been obtained. Making exposures during normal breathing tended to increase this reduction, as the studies on the effect of deep breathing show. But, as has been pointed out, photographing in deep inspiration occasions an error in the other direction; it permits an increase in the volume of blood in the heart, and, consequently, in the area of its shadow, especially in the initial phase when the breath is held. Caution in respect to two other points should be expressed in considering the significance of this outline for clinical purposes. First, the line beginning at the junction of the right border with the diaphragm follows along the right border and passes beyond to the point where it joins the upper extremity of the left border, including within the outline most of the auricles and portions of the great vessels. This line does not follow or represent the ventricles. Second, the line joining the lower ends of the two borders, about the precise drawing of which, as has been said, there is the greater difficulty, is concerned with outlining the margin of one ventricle—the right ventricle. Of the entire cardiac shadow, therefore, only the line of the left border, and indeed, only a portion of this line, accurately follows a ventricular structure. It is, of course, appreciated that what has been said of the line of the right border applies to the value of the transverse diameter as well as to that of the area of the completed outline. No attempt is made at this time to estimate the precise importance of these points; they are matters which require consideration in the study of the abnormal heart.

Although the results depend on these variable factors, the curves (Fig. 4) based on the averaged observations of the area of the heart's shadow, show surprising similarities. The standard curve constructed according to Bardeen's formula is shown for comparison. The lowermost curve represents the averages of the observations obtained in this study. Except for the single observation at 55 kg., the curve lies below the standard. This position is expected in part, because the measurements were made from plates taken in the standing position. Curves based on the measurement of shadows obtained, as was the standard curve in the sitting position, have higher values. Bar-

FIG. 4.—The Area of the Cardiac Shadow.—The ordinates give the area in sq. cm.; the abscissae the body weight. Below the curves is stated at each weight the number of men examined at that weight, on which the average is based. The vertical lines represent the range of observations at that weight; the short oblique lines which cross these indicate the measurement of each case examined. Opposite the short lines is placed the height of that individual. The lowest curve represents the average measurements actually observed in the standing position. The curve above and parallel with this is a corrected curve, giving the estimated area in the sitting position, 7.3 per cent. being added to the lower curve. For comparison the standard curve of Bardeen (the third curve from below upward) is given. The uppermost curve gives the average height of the men examined.

deen estimates the reduction in size resulting from changing from the
prone to the sitting position to be from 5 to 7 per cent., and the reduc-
tion from the prone to the standing position, 13.3 per cent.   From the
sitting to the standing position, a reduction, therefore, of 7.3 per cent.
is expected.   A correction of 7.3 per cent. was, therefore, made.   The
curve so corrected approximates the standard curve.   The corrected
curve then exceeds the standard curve eleven times (53, 55, 56, 57, 59,
62, 65, 68, 69, 72, 77 kg.); it falls below it seventeen times (54, 58, 60,
61, 63, 64, 66, 67, 70, 71, 73, 74, 75, 76, 78, 83, 86 kg.).

It is important also to consider the range of observation at each
weight from which the average curves are constructed, especially in
applying data to the problem of the individual case.   In the present
study (Fig. 4 and Table 8) the greatest range is 64 sq. cm. at 74 kg.
(seven cases); the smallest is 2.0 sq. cm. at 77 kg. (two cases).   At
eight weights (53, 57, 58, 60, 73, 77, 78, 83 kg.) the range is less than
20 sq. cm.; it is between 21 and 30 sq. cm. in six instances (54, 56,
59, 61, 70, 75 kg.); between 31 and 40 sq. cm. in five instances (62, 65,
66, 69, 72 kg.); between 41 and 50 sq. cm. in five instances (63, 64,
67, 68, 71 kg.) and once it is 66 sq. cm. (74 kg.).   But where the
range is greatest, as at 74 kg., four of the seven cases actually lie
within 5 sq. cm. (Table 9).   At other weights, likewise where the range
is wide, the occasional case, rather than the greater number, is respon-
sible for the wide limits.   The range does not appear to be influenced
by the weight (Table 10).   For comparison the data of Dietlen and
Schieffer are added.

A comparison of this result with the results obtained by others is
instructive.   The data published by Dietlen,[10] Schieffer,[11] Bardeen,[8]
and Smith,[6] have, therefore, been arranged in curves in the same way.
Dietlen's data include measurements of both soldiers and of male
civilians.   Separate curves for these two classes have been made.
Arranged in this manner (Fig. 5), Bardeen's curve being included for
comparison, the curve for soldiers coincides with the standard twice
(54, 67); it lies above it thirteen times (52, 53, 56, 58, 59, 61, 63, 66,
68, 69, 71, 74, 76 kg.); and below it eight times (55, 60, 62, 65, 72,
78, 83 kg.).   On the whole, therefore, it lies above rather than below.
The curve for male civilians coincides with the standard twice (53,
63 kg.); it lies above it nine times (55, 56, 57, 58, 59, 61, 69, 71, 75

TABLE 8.

Body Weight According to Range of Cardiac Area.

Range, Sq. Cm.	Weight in Kg.			Range, Sq. Cm.	Weight in Kg.		
	Cohn	Schieffer	Dietlen		Cohn	Schieffer	Dietlen
66	74	—	—	24	59	61	63
62	—	63	—	23	75	—	—
49	64	—	—	22	54	63	—
46	63	—	—	21	61	—	—
45.	67, 71	—	—	20	—	55	64, 70
42	—	60	—	19	—	—	53, 55, 78
41	68	—	—	18	58	64, 71	56
40	72	—	—	17	—	75	66
39	69	—	—	16	—	—	54, 72
38	—	69	—	15	—	57	—
36	—	—	52	14	60	—	—
34	66	62, 56	—	11	—	59	59
33	—	65, 66	—	10	57	—	—
32	—	68	—	9	73	—	—
31	62, 65	—	—	8	53	—	—
29	—	—	60, 62	7	83	—	84
28	56	70	58	5	—	52	—
27	—	—	57, 61, 68	3	78	—	71
26	70	58	65	2	77	—	—
25	—	67	—				

TABLE 9.

Range of Cardiac Area of the Greater Number of Cases at Weights where the Greatest Ranges Occurred.

Range, Sq. Cm.	Weight, Kg.	Range of Greater Number of Cases
66	74	4 of the 7 cases within 5 sq. cm.
49	64	5 of the 10 cases within 10 sq. cm.
45	71	5 of the 8 cases within 17 sq. cm.
45	67	6 of the 10 cases within 20 sq. cm.
46	63	7 of the 11 cases within 15 sq. cm.
41	68	5 of the 10 cases within 8 sq. cm.
40	72	6 of the 9 cases within 12 sq. cm.
40	69	7 of the 12 cases within 18 sq. cm.
34	66	Scattered through entire range
31	65	5 of the 9 cases within 10 sq. cm.
31	62	3 of the 6 cases within 4 sq. cm.
28	56	2 of the 4 cases within 9 sq. cm.

kg.); and below it thirteen times (54, 60, 62,.64, 65, 66, 68, 70, 72, 78, 80, 84, 85 kg.). This curve on the whole, then, lies below it. The soldiers' curve accordingly represents slightly larger hearts than that for civilians. And it exceeds the curve resulting from the present study. With three exceptions Schieffer's curve (Fig. 6) lies uniformly above the standard and represents hearts larger than those in Dietlen's curve. Smith's curve (Fig. 7) follows the standard closely. It coincides with it seven times, falls below it twenty-two times and exceeds it once.

FIG. 5.—The Area of the Cardiac Shadow Based on Dietlen's Tables.—Ordinates and abscissae as in Curve 4. Below the curve is stated at each weight the number of soldiers and of civilian males examined at that weight, on which the average is based. The vertical lines represent the range of observations at each weight; o————————o represents the range for civilians; x————————x represents the range for soldiers. The standard curve of Bardeen is given for comparison.

Interesting as is the comparison of the present curve with Bardeen's standard and the other curves, of greater importance is a consideration of the range. The ranges found by Dietlen extend from 3 to 36 sq. cm. (Fig. 5 and Table 8), and those found by Schieffer from 5

to 62 sq. cm. (Fig. 6 and Table 8). The widest range given by Smith is 11.0 sq. cm. (Fig. 7). It would be interesting to know the range of observed areas at each weight in Bardeen's cases, but this information is not given in his papers. He gives, however, the average percentage of divergence from the standard curves for each 10 kg. of body weight for athletes. Between 51 and 60 kg. the deviation of the average area was 4.6 per cent. (B, Fig. 8) below the standard; between 61 and 70 kg. it was 4.4 per cent. above; between 71 and 80 kg. it was 2.7 per
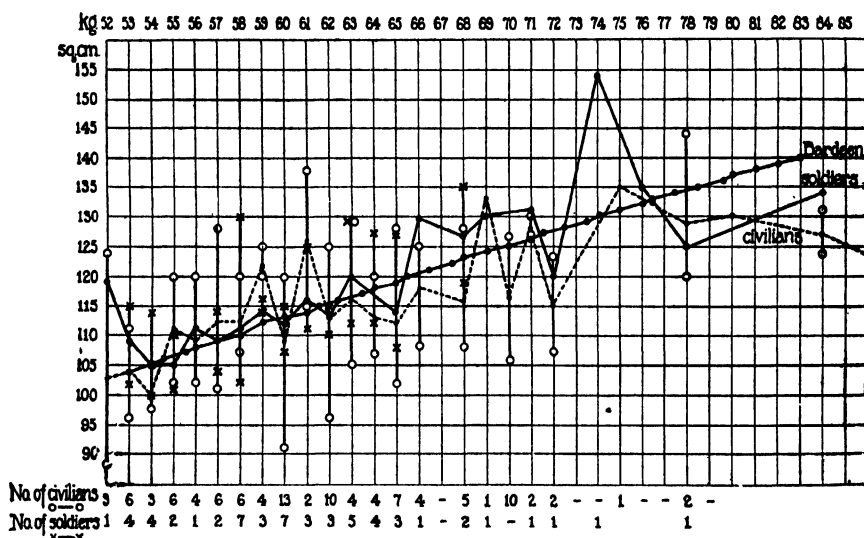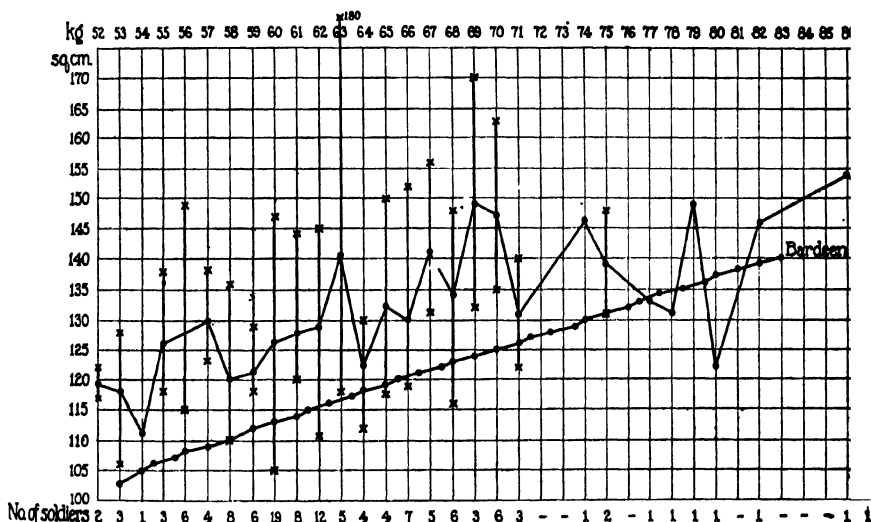

FIG. 6.—The Area of the Cardiac Shadow Based on Schieffer's Table.—Ordinates and abscissae as in Curve 4. Below the curve is stated at each weight the number of soldiers examined at that weight, on which the average is based. The vertical lines represent the range of observations at each weight. The standard curve of Bardeen is given for comparison.

cent. above; and between 81 and 90 kg. it was 2.9 per cent. below. For comparison the deviations from the standard found in the study now presented are arranged in the same manner (C, Fig. 8). The percentage is based on the curve corrected for position of the body. For corresponding weights they are 6.6 per cent. above, coincident with the standard, 6.8 per cent. below, and 13.8 per cent. below.
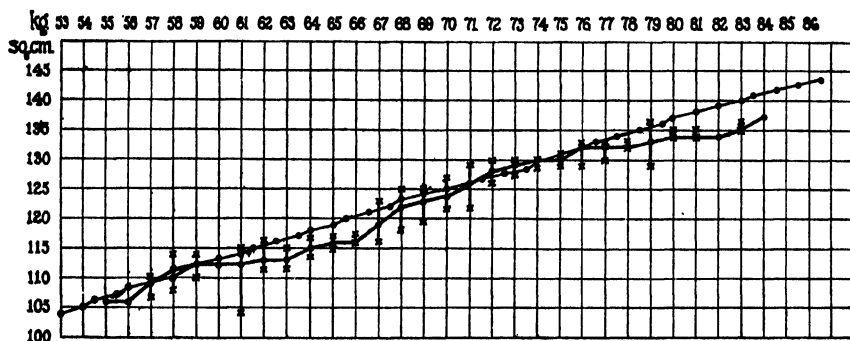
FIG. 7.—The Area of the Cardiac Shadow Based on Smith's Curve and Tables.—Ordinates and abscissae as in Curve 4. The vertical lines represent the range of measurements at each weight. The standard curve of Bardeen is given for comparison.
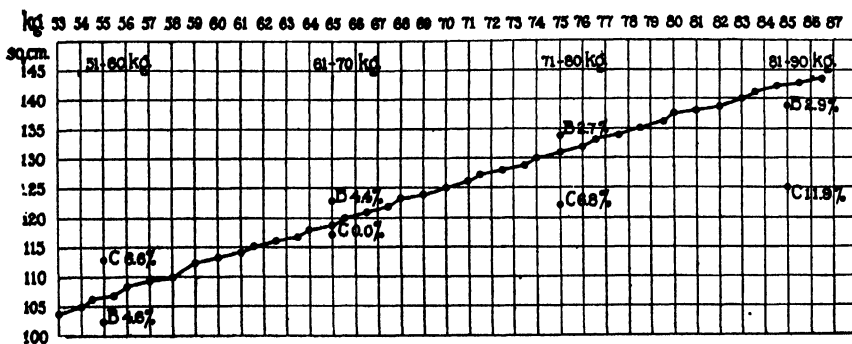
FIG. 8.—The standard curve of Bardeen is given. The average areas of the men examined at weights between 51 and 60, 61 and 70, 71 and 80, 81 and 90 have been inserted for the series now presented (C) and for the figures published by Bardeen (B). Opposite each is given the percentage deviation from the standard curve.

TABLE 10.

Range of Cardiac Area According to Body Weight.

Weight, Kg.	Range		
	Cohn	Schieffer	Dietlen
52	..	5 sq. cm.	36 sq. cm.
53	8 sq. cm.	22	19
54	22 sq. cm.	1 case	16
55	1 case	20 sq. cm.	19
56	28 sq. cm.	34	18
57	10	15	27
58	· 18	26	28
59	24	11	11
60	14	42	29
61	21	24	27
62	31	34	29
63	46	62	24
64	49	18	20
65	31	33	26
66	34	33	17
67	45	25	..
68	41	32	27
69	39	38	1 case
70	26	28	20 sq. cm.
71	45	18	3
72	40	..	16
73	9	..	..
74	66	1 case	1 case
75	23	17 sq. cm.	1 case
76	1 case	..	1 case
77	2 sq. cm.	1 case	..
78	3	1 case	19 sq. cm.
79	..	1 case	..
80	..	1 case	1 case
81	..	..	..
82	..	1 case	..
83	7	..	1 case
84	..	..	7 sq. cm.
85	..	..	1 case
86	1 case	1 case	..
87	..	1 case	..

The curves, then, all show a progressive, even if irregular, increase in the area of the heart's shadow. The usefulness of so-called standard curves, constructed from observed averages, depends, however, on the closeness with which the measurements of the individuals observed, approximate to these curves. Here there is a difficulty, for the wide range of the measurements reduces the value of the average figures. This difficulty apparently does not depend on the technic nor on the individual observer, for it appears in the curves of each.

It has been shown by Dietlen and others that the heart increases in size with the body height. This result is expected because of the usual relation of height to weight. In general therefore, the curves plotted for weight and for height should be similar. The heights of

TABLE 11.

Range of Height at Various Body Weights.

Height, Cm.	Weight, Kg.	Height, Cm.	Weight, Kg.	Height, Cm.	Weight, Kg.
21	60	14	70, 72	9	59
20	74	13	65	7	58
18	66	12	57	5	53, 73
' 71	64	11	63, 68, 71	3	78, 83
15	56, 61, 67, 69	10	62, 75	2	54, 77

these 161 soldiers have been averaged and plotted at each kilogram of weight (Fig. 4). At 53 kg. the height is 153 cm. ' From here the curve ascends not quite in a regular fashion to 83 kg. where the height is 180 cm. The height of each individual examined has also been introduced in the curve (Fig. 4) on the vertical lines representing range, opposite the point indicating the area of the cardiac shadow of each individual. The heights at each weight as the curve shows vary through an extended range (Table 11). Under these circumstances, the suggestion quoted by Schieffer that the area is numerically equal to the height less 50, is scarcely tenable.

Finally, the relation of the angle of cardiac inclination to other measurements has been studied, but there appears to be no rule relating this to the weight, height, or to any other measurement. The introduction of the use of the angle to express what is now indi-

cated by the phrases "transversely lying heart" or "vertically hanging heart" or "pendulous heart" may, however, simplify reference to this phase of physical examination.

SUMMARY AND DISCUSSION.

The attempt was made in this study to learn the size, in comparison with that of civilians, of the hearts of soldiers who had undergone the exertion of actual warfare. The data of soldiers available for comparison are those of Dietlen, Schieffer, and Meakins and Gunson; and for civilians those of Dietlen. Dietlen's curve for area in general resembles Bardeen's standard; Schieffer's exceeds it; the one now presented is a little below it. The differences among the three are probably due to differences in technic of the observers. For transverse diameters, Dietlen's curve lies above Bardeen's standard, the present one lies below it. Schieffer's curve is higher than Dietlen's. If allowance were made (13 per cent.) for the differences between the lying and the standing position, the present curve would exceed Dietlen's and equal Schieffer's. The curves for area and transverse diameters given by Dietlen for civilians differ slightly, but not materially, from those of soldiers. The curves, therefore, do not indicate that soldiers exposed as were these, exhibit larger hearts than normal individuals. Against this conclusion must be set the post-mortem figures given by Karsner[12] for the hearts of British soldiers who were 27 years of age, and who had served twenty-two months. These were larger than the controls. If the requirements set by Karsner are sound, enlargement may not be expected in our soldiers whose average age was 24 and whose length of service was usually less than twenty-two months. Furthermore, the examinations here reported were conducted in May, 1919, six months after the cessation of hostilities. Although within that period many had undergone severe exertion, the amount of work they performed probably decreased. A diminution in size of the heart from the maximum reached in November, 1918, may have taken place, but no data are available to indicate whether decreasing the amount of work affects the subsequent size of the heart.

12. Karsner, H. T.: Acute Endocarditis Following War Wounds, Including Notes of Heart Weight and Arteriosclerosis in Soldiers, Arch. Int. Med. 22: 296, 1918.

The technic differed from that usually employed in that the plates were taken for the most part during normal inspiration. There is a twofold reason for this; first, it avoids the increase of the heart's size which occurs during deep inspiration. A more nearly normal heart is photographed. Second, it is more likely to provide a standard against which the pathologic heart may be measured. For, as has been pointed out, in increasing heart failure, it becomes progressively more difficult to utilize the method which obliges the subject to breathe deeply and to hold the breath. The technic here employed does not require holding the breath and avoids the artificially large heart. There would probably be no objection to this alteration in technic were it not that exposures made in deep breathing lighten the lung fields, so permitting a sharper image of the heart, and expose in certain individuals, especially those in whom the angle of inclination of the heart is small, a greater extent of the heart's outlines. In the last point there is force. It is not, however, so cogent a reason as it appears to be, for deep breathing aids in locating the apex of the heart and in drawing the long diameter and the area. But these measurements are not superior in value to the transverse diameter, for this diameter increases with the body weight as do the others, it shows no greater variation at each weight, and it can be taken accurately without reference to respiration, and in any case involves no uncertainty such as is necessary in locating the apex and drawing the line indicating the border of the right ventricle.

The value for the present of so-called standard and average curves of cardiac measurements for use in the clinic is questioned. For this reason the range of the measurements for given weights has been emphasized. The difficulty in the use of these curves is illustrated in comparing the cardiac area of a soldier weighing 54 kg. with that of one weighing 86 kg. The areas of the two (Curve 4) are 108 sq. cm. and 110, respectively. And the difficulty is illustrated again, in comparing the cardiac area of a soldier weighing 56 kg. with one weighing 83 kg., the areas of both being 120 sq. cm. Similar comparisons can be made in Dietlen's and Schieffer's curves (Figs. 5 and 6). For the clinic curves of this nature do not solve the problem of supplying adequate criteria for judging the normality of a given heart. There is, still, necessity for more than a single

criterion, such as size. In other connections these curves have a signal usefulness; they may, indeed, serve as bases for studies, in which, combined with other factors, satisfactory criteria for clinical use will be evolved.

CONCLUSIONS.

1. In normal breathing the difference in the size of the heart during inspiration and expiration may be neglected.

2. The use of the transverse diameter of the heart shadow is a satisfactory measurement. It is as useful as and less uncertain than the long diameter or the area.

3. The range of the observed measurements interferes with the usefulness for the clinic of standard and average curves.

4. The hearts of soldiers examined under conditions stated are not larger than those of normal individuals.

THE EFFECT OF ACUTE YELLOW ATROPHY ON METABOLISM AND ON THE COMPOSITION OF THE LIVER.

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In the present paper we report chemical analyses of the blood and urine made in a case of acute yellow atrophy for three days before death, together with analysis of the liver removed at necropsy three hours after death. The study was directed especially toward the nitrogen metabolism and the acid-base balance of the patient. Besides other determinations, including those of urea, quantitative estimations of the amino-acid nitrogen in the urine, blood and liver were performed for the first time in such a case, so far as we can ascertain.

The results are of interest in two connections: (1) the pathologic metabolism of acute yellow atrophy, and (2) the rôle of the liver in the normal handling of nitrogenous products. The condition is one in which the liver as a functioning organ suffers almost complete, if not complete, destruction, without similar apparent injury to other organs; and the failure in yellow atrophy of definite steps in nitrogenous metabolism may be taken as an indication of the possibility that such steps are normally achieved either by the liver or with its aid.

Review of Literature.

Since Frerichs[1] (1861) established the presence of leucin and tyrosin in both the liver and urine in acute yellow atrophy, there has been basis for the belief that in this disease the liver proteins are digested to amino-acids, which are excreted as such in the urine. Schultzen and Riess[2] (1869) are quoted as declaring that the presence of leucin and tyrosin in the urine indicates acute yellow atrophy as definitely as the presence of albumin indicates nephritis. In nearly

1. Frerichs: Klinik der Leberkrankheiten, Braunschweig, 1861.

2. Schultzen and Riess: Hammarsten's Lehrb. d. Physiol. Chem., Ed. 8, Wiesbaden, 1914, p. 705.

all cases reported, however, the amino-acids have been identified only by microscopic examination of the crystals, and Röhmann[3] (1888) with the utmost care was unable definitely to identify either leucin or tyrosin in the antemortem urine in a case terminating fatally. Röhmann explained the failure of these substances to appear in some cases of yellow atrophy by the hypothesis that amino-acids are excreted only when formed at a rate faster than they can be destroyed in the body. In his case an aromatic acid, probably p-hydroxyphenyl lactic acid, formed by replacing the NH_2 group of tyrosin by an OH (Kotake,[4] 1910), was obtained from the urine. Its presence indicated that the body could still deaminize tyrosin, even though the product of deamination failed of further combustion.

Neuberg[5] (1904) claimed to have obtained from the blood serum in a fatal case tyrosin, leucin and lysin, the three amino-acids together amounting to about 6 gm. per liter of blood. This indicated the presence of so great an amount of amino-acids in the blood, that the investigators believed they must be due to autolysis of the muscles as well as the liver. At the same time, the nitrogen distribution in the urine was normal, 76 per cent. of the total nitrogen being in the form of urea. The results of the blood and urine analyses are both so unusual and so completely at variance with each other (i.e., tremendous amounts of amino-acids in the blood with no apparent disturbance of the nitrogen distribution in the urine) that without confirmation their acceptance as accurate in a quantitative sense is difficult.

Wells[6] (1907) has published the most complete examination of the liver in yellow atrophy. He submitted the noncoagulable, water-soluble extract to Fischer's ester process for isolation of the monoamino-acids, and to Kossel's procedure for the diamino-acids, and identified histidin, lysin, tyrosin, leucin, glycin, alanin, prolin, glutaminic acid and aspartic acid, the total amount being 8.67 gm. from the entire liver. To judge from the usual losses by the ester method, the amounts present were probably twice as great as those actually obtained. Wells proved beyond doubt that the liver in acute yellow atrophy does contain considerable amounts of the various amino-acids yielded by proteins on hydrolysis. That previously only leucin and tyrosin were, as a rule, found was obviously due to the fact that these are the amino-acids which are most easily obtained by crystallization.

Even the results of Wells, however, did not entirely solve the problem from a quantitative standpoint. Van Slyke and Meyer[7] (1913–14), and Abel, Rowntree and Turner[8] (1914) showed that amino-acids in considerable amounts are normal

3. Röhmann: Berlin klin. Wchnschr. 861, 882, 1888.
4. Kotake: Z. physiol. Chem. 65: 397, 1910.
5. Neuberg and Richter: Deutsch. med. Wchnschr. 30: 499, 1904.
6. Wells: J. Exper. Med. 9: 627, 1907.
7. Van Slyke and Meyer: J. Biol. Chem. 16: 197, 1913–14.
8. Abel, Rowntree and Turner: J. Pharmacol. Exper. Therap. 5: 275, 1914.

constituents of the blood and tissues. The conceptions of the changes in liver atrophy, and their significance in regard to liver function, therefore, became dependent on the question whether or not the amounts of amino-acids formed and excreted in acute yellow atrophy vary significantly from the normal. In the present paper we are able to answer this question in the affirmative, and thereby confirm the older conceptions of the metabolism in this disease and, we believe, place them on a more complete experimental basis.

Report of Case.

The patient, female, aged 29 years, had been well to within one day of admission to the hospital. Her first symptoms were nausea and vomiting, at first mild, but persistent and gradually increasing in intensity. She had no chills, fever or pain. There was slight headache. Bowels were slightly constipated. The vomitus consisted of greenish, bile-tinged fluid.

On admission to the hospital, physical examination showed temperature was 100; pulse 75; respirations 20. Sclerae showed a very slight but definite icterus. Heart and lungs were negative. Abdomen negative. Liver and spleen not felt. Skin negative; no jaundice.

Second Day.—Patient continued vomiting all day; no pain. Temperature 99.6. Definite jaundice had developed over the entire body.

Third Day.—Vomiting persistent and intractable. She was unable to retain even water by mouth. Jaundice much more marked.

Fourth Day.—She was very weak and restless. No pain; no headache. All measures to allay the continuous vomiting were unsuccessful. Jaundice deeper. The abdomen, heart and lungs continued negative. Pulse 90, respirations 20, temperature 100.0 F.

Fifth Day.—Patient vomited continually. Later she gradually sank into coma. No convulsions. Sclerae and skin of a deep greenish yellow color. Pupils were widely dilated but equal, and reacted slightly to light. Fundi oculi showed nothing. Blood pressure, 155/70. Abdomen soft. Liver and spleen not felt. Liver dulness extended from fourth rib to just below the costal margin. All extremities quite rigid, no Kernig. Exaggerated knee jerks on both sides. Marked double ankle clonus. Definite Babinski on the left side, none on the right. No Chvostek or main d'accoucheur.

Lumbar puncture: No increased pressure. Fluid clear, cells 5 per c.c.; spinal fluid urea nitrogen, 0.073 gm. per liter. No bile (Smith's test).

In the late afternoon rigidity of the extremities and Babinski disappeared, and the knee jerks became less marked. No convulsions at any time or further evidence of tetany. No subcutaneous hemorrhages; at point of pressure, however, by the bed clothes there were small areas of ecchymosis. No diminution of the liver dulness made out during the day.

Sixth Day.—Patient continued comatose, temperature and pulse rapidly rising. At 8 a. m., temperature was 108; pulse, 145; respirations, 44. No decrease of liver dulness made out.

Patient died at 8:33 a. m.

Wassermann completely anticomplementary.

Necropsy Findings.—Macroscopic: Skin was deeply jaundiced, of a yellowish green color. Lungs were normal, except for a few scattered, hemorrhagic patches. Heart ·was negative. Abdomen: Omentum and the mesentery showed everywhere numerous hemorrhagic areas from 3 to 5 cm. in diameter. Stomach and intestines, gallbladder, pancreas, kidneys, suprarenals and uterus were normal. Ovaries contained few cysts. Spleen not enlarged; extremely dark in color. On section it was quite soft. Cut surface was a very dark purplish red. Malpighian bodies were not well made out.

Liver was quite markedly diminished in size. It was not found fallen away from the anterior abdominal wall. Its upper border corresponded with the fourth rib; its lower border to the costal margin. Weight, 1,000 gm. Capsule was smooth, and the parenchyma shining through appeared quite yellowish. Liver substance was extremely friable and cut with great ease. On section, the liver surface showed at the center of each lobule a dark reddish area surrounded by a lighter yellow zone. This appearance was quite uniform throughout the entire liver, except that there were occasional patches from 5 to 10 mm. in diameter of a darker red, and these areas were sunken below the general surface. Other areas, larger in size, were yellowish and studded with small reddish points representing the centers of the lobules. In these yellowish patches the centers of the lobules were smaller and less numerous than in other parts of the liver. There were no areas which suggested adenomatous hyperplasia.

Microscopic: Only the liver and kidney appeared abnormal under the microscope. The uterus, spleen, suprarenals, pancreas, lungs and bladder were examined, with negative results.

Kidney: There are scattered throughout the cortex small areas of hemorrhage between the tubules and in Bowman's capsule. Glomeruli appear quite normal, but the tubules show here and there a moderate degree of fatty infiltration and cloudy swelling. Otherwise the parenchyma was normal.

Liver: Universally, the histologic picture is characterized by an extensive destruction. Throughout most of the liver, cells are represented by only faint outlines with completely or partially disintegrated nuclei. In some areas the liver cells are represented only by a structureless debris, while in others the outlines of the cell bodies can be faintly made out still in lobular arrangement. There is an extensive fatty infiltration. These changes involve practically all of the lobule, but are most extensive at the periphery. At the center of each lobule the bile duct epithelium is practically intact, and here may be found quite generally very few liver cells in a better state of preservation. Some of the bile ducts are surrounded·by well preserved liver cells arranged in strands. Scattered throughout, mainly at the periphery of the lobules, are areas of blood extravasation.

Analytical Methods.—Urine Analyses: Chlorids were titrated by the Volhard method. Urea was determined by Van Slyke and Cullen's[9] (1917) modification of Marshall's urease method, using Squibb's urease prepared from Jack beans, ammonia by the aeration technic described by Van Slyke and Cullen. The total amino-acid nitrogen was determined as described by Levene and Van Slyke[10] (1912), the creatinin by Folin's[11] (1905) method, and the uric acid by Folin and Shaffer's[12] (1901) method. Total acetone bodies were estimated by Van Slyke's[13] (1917) gravimetric method. The titratable acid was determined according to Folin[14] (1903) by titration with phenolphthalein in the presence of potassium oxalate.

Blood Analyses: The urea was determined as described by Van Slyke and Cullen[15] (1914), the amino-acid nitrogen by Van Slyke's nitrous acid method as described by Whipple and Van Slyke[16] (1918). The plasma bicarbonate was estimated by the carbon dioxid capacity method of Van Slyke and Cullen[9] (1917).

Liver Analysis.—Water content: Three samples of from 2 to 3 gm. weight each from different lobes of the liver were dried in glass dishes at 110C. to constant weight. The results were 71.6, 71.6 and 71.9 per cent. water.

Total Nitrogen.—The dried samples used for water. determination were Kjeldahlled. The results were 8.0, 8.36 and 9.0 per cent. of nitrogen calculated on the dried samples, and 2.28, 2.34 and 2.51 per cent. calculated on the fresh samples, an average of 2.38 per cent. of the fresh substance, or 8.45 per cent. of the dried. Using the approximate factor 6.25 for conversion of nitrogen figure into protein figure, this would indicate that 5.3 per cent. of the dry substance was protein.

Fat.—Three samples of from 6 to 12 gm. from different lobes were let stand over night with 100 c.c. of 95 per cent. alcohol each. The alcohol was poured off, the liver samples were minced, and were extracted with ether in a Soxhlet apparatus. The alcohol extracts were concentrated nearly to dryness, taken up with ether, and combined with the ether extracts. The latter were concentrated to dryness, and taken up with petroleum ether in weighed flasks. After the petroleum ether had been mostly removed on the water bath, the flasks were dried in an evacuated desiccator over sulfuric acid to constant weight. The results were 12.7, 13.8 and 14.0 per cent. of fat, an average of 13.5 per cent. of the fresh liver, or 47.7 per cent. of the dry substance.

9. Van Slyke and Cullen: J. Biol. Chem. 30: 289, 1917. Van Slyke, Stillman and Cullen: J. Biol. Chem. 30: 401, 1917. Stillman, Van Slyke, Cullen and Fitz: J. Biol. Chem. 30: 405, 1917.

10. Levene and Van Slyke: J. Biol. Chem. 12: 301, 1912. Van Slyke: J. Biol. Chem. 16: 125, 1913–14.

11. Folin: Am. J. Physiol. 13: 48, 1905.

12. Folin and Shaffer: Ztschr. physiol. Chem. 32: 552, 1901.

13. Van Slyke: J. Biol. Chem. 32: 455, 1917. Van Slyke and Fitz: J. Biol. Chem. 32: 495, 1917. Palmer and Van Slyke: J. Biol. Chem. 32: 499, 1917.

14. Folin: Am. J. Physiol. 9: 265, 1903.

15. Van Slyke and Cullen: J. Biol. Chem. 19: 211, 1914.

16. Whipple and Van Slyke: J. Exper. M. 28: 213, 1918.

Extraction of Nonprotein Nitrogen.—Three samples, totalling 303 gm., were cut with scissors into pieces which were dropped into 1.5 liters of boiling water slightly acidified with acetic acid.   After the tissues were coagulated, the water was decanted, the pieces were minced, and the extraction with hot water was repeated three times.   The decanted extracts were filtered through glass wool, then mixed with 50 gm. kaolin, and filtered through paper with suction, the residue being washed with hot water.   The clear amber filtrate was boiled down in an enamelled ware vessel to about 500 c.c., and poured into three volumes of absolute alcohol to complete the removal of proteins.   The next day the precipitate which formed was filtered off, and washed with 80 per cent. alcohol.   The filtrate was concentrated under reduced pressure and brought to a volume of 150 c.c.

Free Amino Nitrogen.—Two c.c. portions of extract were used for the determination of amino nitrogen by the nitrous acid method of Van Slyke[17] (1912), the reaction being continued for 3.5 minutes at 22 C.   The volume of gas yielded in three determinations was 9.55, 9.50 and 9.65 c.c. at 22 C., 761 mm., the average, 9.57 c.c., indicating 5.40 mg. of amino nitrogen, or 134 mg. per 100 gm. of fresh liver.

Peptid Nitrogen.—Five c.c. samples of the extract were mixed in test tubes with 5 c.c. portions of concentrated hydrochloric acid and heated for twenty-four hours at 100 C.   The hydrochloric acid was driven off on the water bath, and the residue diluted to 20 c.c., of which 2 c.c. portions were used for the amino nitrogen determination.   The determinations yielded 3.72, 3.67 and 3.63, average 3.67 c.c. of nitrogen at 22 C., 759 mm., indicating 206 mg. of amino nitrogen per 100 gm. of fresh tissue.   Subtracting the 134 mg. present as per amino nitrogen before hydrolysis, leaves 72 mg. as peptid nitrogen, freed by hydrolysis.

Urea and Ammonia Nitrogen.—These determinations were made as in urine (Van Slyke and Cullen,[9] 1917), 5 c.c. of extract being used for urea and the same amount for ammonia.   The results of triplicate determinations indicated 14.8 mg. of urea nitrogen and 34.5 mg. of ammonia nitrogen per 100 gm. of fresh tissue.

Creatinin.—Folin's method was used as for urine[11] (1905), except that the final dilution was less.   Five c.c. of the extract were mixed with 15 c.c. of saturated picric acid solution, and 5 c.c. of 10 per cent. sodium hydroxid.   At the end of five minutes the solution was diluted to 50 c.c. and read against a dichromate standard.   The result, which may be somewhat high because of the relative concentration of sodium picrate, indicated 3.3 mg. of creatinin nitrogen per 100 gm. of fresh tissue.

Creatin Plus Creatinin.—Five c.c. of extract were heated at 100 C. with 5 c.c. of N hydrochloric acid for three hours.   The hydrochloric acid was neutralized with concentrated sodium hydroxid, and 5 c.c. of 10 per cent. sodium hydroxid in excess were added with 15 c.c. of saturated picric acid.   After five minutes the solution was diluted to 100 c.c. and read against a chromate standard.   The

17. Van Slyke: J. Biol. Chem., **12**: 275, 1912.

reading indicated 17.6 mg. of creatin plus creatinin nitrogen, or 14.3 mg. of creatin nitrogen per 100 gm. of fresh liver tissue. ·

The technic used is similar to that employed by Janney and Blatherwick[18] (1915). The difference is that the above authors used aluminum hydroxid to adsorb uncoagulated protein (presumably gelatin), while we used kaolin. Creatinin under proper conditions is adsorbed by kaolin (Greenwald,[19] 1918), but we found by control tests that the kaolin preparation used by us in the amounts employed did not remove sufficient creatinin to significantly affect results such as were obtained.

Discussion of Results.

Excretion.—The comatose condition of the patient made collection of complete twenty-four hour specimens impossible, so that conclusions have to be drawn largely on the nitrogen distribution. The abnormalities in the latter during the last two days before death are

TABLE 1.

Salt and Nitrogen Excretion.

Date, 1919	Output C.c.†	Specific Gravity	Bile	NaCl, Gm. per L.	Total N, Gm. per L.	Urea N		Amino-Acid N		NH₃ N		Creatinin N		Uric Acid N		Undetermined N	
						Gm. per L.	Per Cent. of Total N	Gm. per L.	Per Cent. of Total N	Gm. per L.	Per Cent. of Total N	Gm. per L.	Per Cent. of Total N	Gm. per L.	Per Cent. of Total N	Gm. per L.	Per Cent. of Total N
Feb. 28	1,070	1.013	+	....	.....	3.11	....	....	....	0.392							
Mar. 1	250+	1.022	+	1.04	13.90	7.04	47.2	0.61	4.1	0.862	16.2	0.65	4.4	0.185	1.2	4.65	32.7
Mar. 2	411+	1.032	+	0.28	10.94	5.68	51.9	1.75	16.0	1.880	17.2	0.54	2.0	0.128	1.7	0.96	8.1
Mar. 3	130+	1.031	+	1.38	16.64	7.75	46.6	2.22	13.3	1.925	11.6	0.49	2.9	0.417	2.5	3.82	23.0

Tyrosin crystals were obtained from the combined specimens of urine not used for other analyses.

Part of urine was lost on March 1, 2 and 3 because of patient's comatose condition.

the high ammonia ratio, and even more strikingly, the high amino-acid nitrogen and the low proportion of urea nitrogen (Table 1). All three are explainable on the assumption that the liver had lost a part of its ability to transform amino-acid nitrogen into urea nitrogen, one portion being excreted in the form of unchanged amino-acids, and another in the form of ammonia.

18. Janney and Blatherwick: J. Biol. Chem. 21: 567, 1915.
19. Greenwald: J. Biol. Chem. 34: 103, 1918.

The amount of nitrogen eliminated cannot be stated accurately because of the lack of twenty-four hour samples of urine. The probable minimum can, however, be approximately estimated from the creatinin. The creatinin nitrogen excretion of an individual of the patient's size (about 50 kg.) would normally be about 4.40 gm. The twenty-four hour excretions of nitrogen and of ammonia plus titratable acid, estimated in this manner, are given in Table 2. In an acute illness with pathologic tissue loss, the creatinin output might

TABLE 2.

Approximate Twenty-Four Hour Nitrogen and Acid Excretions Estimated from Creatinin Output.

Date	A Creatinin N per L. Mg.	B Proportion of 24 Hr. Excretion in 1 Liter of Urine Sample Obtained, Estimated from Creatinin, A — 400	Estimated Excretion per 24 Hours = Excretion per Liter Urine — B	
			Titratable Acid + NH₃, C.c. 0.1 N	Total N Gm.
March 1........	650	1.62	670	8.6
March 2........	540	1.35	1,530	8.1
March 3........	490	1.22	1,530	13.6

be increased. Even if it remained only normal at 0.40 gm. per day, however, the total nitrogen excretion on March 1, 2 and 3 would be calculated at 8.6, 8.1 and 13.6 gm., respectively, assuming that the ratio of twenty-four hour total nitrogen to the observed nitrogen per liter is as 0.40 to the observed creatinin nitrogen per liter. The first two figures might be considered normal for a fasting individual (Lusk,[20] 1917) but the last is certainly high and corresponds with the incidence of fever.

Small amounts of acetone bodies were excreted, but in quantities not sufficient to be of significance for the acid-base balance of the body.

The low salt output was presumably due to lack of salt intake, rather than kidney retention.

20. Lusk: Science of Nutrition, Philadelphia, 1917.

TABLE 3.

Acid Excretion and Alkaline Reserve.

Date, 1919	Excretion per Liter of Urine			Total Acetone Bodies, C.c. 0.1 N	Blood Plasma Bicarbonate CO_2, Volume per Cent.
	NH₃, C.c. 0.1 N	Titratable Acid, C.c. 0.1 N	Acid + NH₃, C.c. 0.1 N		
February 28...............	280	92.4	372		
March 1..................	616	460	1,076	64	
March 2..................	1,343	719	2,062	180	96.5
March 3..................	1,375	496	1,871	...	65.4
March 4..................	.....	...	.....	...	49.0

Blood Analyses.—The analyses of the blood, like those of the urine, indicate a loss of deaminizing function in the body (Table 4). The amino nitrogen contents on the last two days are respectively two and three times as great as the 7 to 8 mg. per 100 c.c. which represent the average normal (Bock,[21] 1917). The apparent explanation is that the liver had lost the ability to transform amino-acids into urea, an

TABLE 4.

Urea and Amino-Acid Nitrogen in the Blood.

Date, 1919	Urea N, Gm. per L.	Amino-Acid N, Gm. per L.
February 28....................................	—	—
March 1.......................................	—	—
March 2.......................................	0.123	0.140
March 3.......................................	0.088	0.173
March 4.......................................	0.159	0.263

ability which has been demonstrated in the normal liver by the perfusion experiments of Salaskin[22] (1898) recently confirmed by Jansen (1915), and by the physiological experiments of Van Slyke, Meyer, Cullen and McLean (Van Slyke,[23] 1917).

The source of the amino-acids in the blood in our case was undoubtedly autolyzed tissue protein. The patient, because of the nausea,

21. Bock: J. Biol. Chem. **29:** 191, 1917.
22. Salaskin: Ztschr. f. physiol. Chem. **25:** 128, 1898.
23. Van Slyke: Arch. Int. Med. **19:** 56, 1917.

retained practically no food; the considerable amounts of nitrogen excreted (8 gm. or more per day) must have come from tissues autolyzed under the influence of the fasting and intoxication attending the disease.

The blood urea remains within normal limits. Taken with the continued output of urea in the urine, it shows that the urea-forming function of the body, although diminished, was only partially lost, even on the day before death.

The blood plasma bicarbonate determinations (Table 3) gave a peculiar result on the first observation four days before death. The carbon dioxid capacity of 96.5 per cent. would indicate an abnormally high alkaline reserve. Unfortunately, the observation was not repeated on that day, and we are uncertain whether an increased blood bicarbonate actually existed, or whether there was an error in the determination. The carbon dioxid estimations were done in duplicate, but the carbon dioxid capacity might have been raised in vitro by contamination of the centrifuge tube or equilibrating funnel with alkali. That this may have occurred is made more probable by the fact that the excretion of titratable acid and ammonia on this day was above the usual normal limits.

'On the last two days before death, there was a high excretion of titratable acid and ammonia, about double the normal, and on the day before death the plasma bicarbonate fell slightly below normal (49 per cent. of carbon dioxid). These facts indicate a definitely accelerated formation of acids in the organism, but neither the acid excretion nor the plasma bicarbonate indicates an acidosis sufficient to have in itself a definite effect on the patient's condition. (Compare Van Slyke,[24] 1917 [b]). In connection with the increased acid production it is of interest to note that Röhmann[3] (1888) obtained unusual aromatic acids and considerable amounts of sarcolactic acid from the urine in a case of yellow atrophy.

Composition of the Liver.—The most striking change in the liver was the marked loss of substance, which is usually noted in acute atrophy (Wells,[6] 1907). The liver weighed only 1,000 gm. instead of the 1,800 gm., which, for an individual of the patient's size, is normal, according to Frerichs[1] (1861).

24. Van Slyke: J. Biol. Chem. 32: (b) 455, 1917.

The water content of 71.7 per cent. (Table 5) shows no increase over that of the normal liver analyzed by Wells, in fact is less than the latter (76.1 per cent.). In this respect this case differs from most of those in the literature, as the water content usually has been found high in acute yellow atrophy.

The fat content of 13.5 per cent. is decidedly high, the normal being quoted by Wells[6] (1907) as about 3 per cent. The livers of acute atrophy of which analyses are reported in the literature (Wells, 1907, 1908) have been found to vary between from 2.0 and 8.7 per cent. in fat content, while those of phosphorus poisoning and fatty degeneration show from 25 to 30 per cent. In our case of acute atrophy the difference from the high fat values of phosphorus poisoning and fatty degeneration is much less striking than in any of the instances reported. It may be that the unusually rapid progress, with death a

TABLE 5.

Dry Matter, Fat and Total Nitrogen in Liver.

	Per Cent.	Normal Values, per Cent.[*]	
Water..	71.7	77.6	76.1
Dry matter..................................	28.3	19.4	20.9
Dry matter as fat............................	13.5 ⎱ 28.4	5.0	3.0
Dry matter as protein calculated as N × 6.25......	14.9 ⎰		

* From Wells (1908).

few days after the first symptom, prevented the complete combustion of the fat transported to or formed in the liver. The high fat content in our case is the cause of the normally high content of total solids noted in the preceding paragraph; fat increase makes up for the protein loss.

The "dry matter not fat," which, as indicated by its nitrogen content, was nearly all protein, was much decreased, as it has been in all the reported cases of liver atrophy, whether due to poisoning or to acute disease. In our case the content of solids not fat, or approximately the protein, is 14.9 per cent. instead of the normal 20 per cent. of fresh liver. During the few days of the disease, the liver, therefore, in losing about 45 per cent. of its weight lost about 60 per cent. of the protein substance.

In the nitrogen distribution in the liver (Table 6) the main point of interest is the high content of amino-acid and peptid nitrogen. Compared with livers of normal dogs, the amino nitrogen, 0.134 per cent. of the fresh tissue, is about three times as great (Van Slyke and Meyer,[7] 1913–14). The observed amino nitrogen content indicates the presence of approximately 13 or 14 gm. of free amino-acids in the 1,000 gm. of liver tissue.

TABLE 6.

Nitrogen Distribution in Liver.

A. Total N	Per 100 Grams Liver, Gm.	Proportion of Total N, per Cent.
Total N...............................	2.380	100.0
Nonprotein N...........................	0.315	13.2
Protein N (by difference)...................	2.065	86.8

B. Nonprotein N	Per 109 Grams Fresh Liver, Gm.	Proportion of Total Nonprotein N, per Cent.
Total nonprotein N.......................	0.3130	100.0
Urea N................................	0.0148	4.76
Ammonia N.............................	0.0345	10.95
Amino N...............................	0.1340	42.50 ⎱ 65.3
Peptid bound N..........................	0.0720	22.80 ⎰
Creatin N..............................	0.0143	4.54
Creatinin N.............................	0.0033	1.05
Undetermined N.........................	0.0421	13.40

Rôle of the Liver in Nitrogenous Metabolism.

The apparent significance of these figures, along with those for the blood and urine, is that the liver protein was autolyzed at a rapid rate to amino-acids and peptids, chiefly the former, and that amino-acids produced by the abnormal autolysis of the liver and the normal autolysis of the rest of the body (other organs were not macroscopically or microscopically degenerated) were turned into urea to the extent of only about 60 per cent. instead of from 85 to 95 per cent. The excreted amino-acids formed as high as 16 per cent. of the total urinary nitrogen instead of the normal 2 per cent.

There was no indication at any time of a tendency for the urea nitrogen output to fall much below 50 per cent. of the total nitrogen. In view of the apparently complete degeneration of the liver cells the data consequently suggest the probability that, although deamination and urea synthesis without the liver are incomplete, nevertheless they can occur to such an extent that the greater part of the nitrogen normally excreted as urea is still in this form. Fiske and Sumner[25] (1914) found that dogs could form urea from injected glycocoll even after the abdominal viscera had been excluded from the circulation. It is uncertain whether a considerable part of the total urea synthesis normally occurs in parts other than the liver, or whether the process is taken up elsewhere only when the liver fails.

It appears that acute yellow atrophy, with the possible exception of fatal phosphorus poisoning, is the only clinical condition in which unusual amounts of amino-acids have been demonstrated to be formed and excreted as such, without change to urea or ammonia.

The most rapid autolysis unaccompanied by liver degeneration does not apparently result in the excretion of amino-acids. For example, we have not found the amino nitrogen of the blood or urine increased during resolution in pneumonia, although this process represents one of the most striking examples known of rapid autolysis in vivo. Nor was any abnormal increase in the blood amino nitrogen observed by Whipple and Van Slyke[16] (1918) in dogs that were intoxicated by proteose or by intestinal obstruction in such a manner that the blood urea was raised by autolysis of body protein in a few hours to form two to five times the fasting level. Such urea changes indicate an amount of tissue digestion seldom met in disease, and not approximated in the apparently localized autolysis of yellow atrophy.

In order that amino-acids in exceptional amounts shall escape deamination and appear in abnormal amounts in the urine, it appears necessary not only that the liver shall be injured, but that its loss of function shall be profound, and, as stated above, such loss of function apparently has been observed in man with certainty only in acute atrophy. We have been unable to confirm the statement of Labbé and Bith[26] (1911) that it occurs in diabetes (Van Slyke and

25. Fiske and Sumner: J. Biol. Chem. **18**: 285, 1914.
26. Labbé and Bith: Progrés méd. **27**: 581, 1911.

Stillman, unpublished results).   It does not occur in the toxemias of pregnancy, despite the marked degenerative changes that occur in the liver (Losee and Van Slyke,[27] 1917).   Levene and Van Slyke[10] (1912) failed to observe it in two cases of cirrhosis.   Chesney, Marshall, and Rowntree[28] (1914) report somewhat increased amino-acid nitrogen in both blood and urine in more than 50 per cent. of a series of cases with apparent liver insufficiency from various causes.   However, the upper limits for amino nitrogen which they assumed were so low (1.5 per cent. of the urine nitrogen, and 3 mg. per 100 c.c. of blood) that they are readily exceeded in normal individuals (Levene and Van Slyke,[10] 1912; 1913–14; Bock,[21] 1917; Cullen, Ellis and Van Slyke,[29] 1915).   The only case in their series with definitely high blood amino nitrogen was one of arsphenamin poisoning, with 12.4 mg. per 100 c.c.

In pathologic liver conditions caused experimentally, it appears to be likewise unusual, unless liver degeneration is almost complete, to find increased amino-acid content in blood or urine.   Whipple and Van Slyke[16] (1918) failed to observe it in the blood of dogs with Eck fistulas (unpublished results).   Levene and Van Slyke[10] (1912) did not find it in the urines of dogs in which Opie and Dochez had caused liver degeneration by phosphorus and chloroform poisoning.   Only Marshall and Rowntree[30] (1915) observed an increase in the blood amino nitrogen (up to 21 mg. per 100 c.c.) shortly before death in dogs poisoned with phosphorus.

In pathologic conditions not involving the liver, Bock[21] (1917) found very markedly increased blood amino nitrogen only in some cases of nephritis, in which amino-acids are retained along with other urinary constituents.

<div align="center">SUMMARY.</div>

An increased excretion of ammonia and titratable acids was observed in the last days of illness, and a fall of plasma bicarbonate to slightly below normal on the day before death.   Even at this time, however,

27. Losee and Van Slyke: Am. J. Med. Sc. 153: 94, 1917.
28. Chesney, Marshall and Rowntree: J. A. M. A. 63: 1533, 1914.
29. Cullen, Ellis and Van Slyke: J. A. M. A. 64: 126, 1915.
30. Marshall and Rowntree: J. Exper. M. 22: 333, 1915.

the deviations from the normal were too small to indicate that acid intoxication was a significant factor in the condition.

The results afford confirmation of a quantitative character for the belief that amino-acids are formed by autolysis in the atrophying liver, and circulate and are excreted as such in unusual amounts.

The excretion of amino-acids did not appear to be due to increase in their rate of formation, for the total protein katabolism was not abnormally or even unusually rapid. The excretion appeared due rather to loss of power to deaminize amino-acids at even an ordinary rate.

A review of the known instances of rapid intra vitam autolyses not involving the liver indicates that tissue waste alone does not cause increase of amino-acids in the blood and urine. In conditions of less profound liver injury (eclampsia) a marked decrease may occur in the proportion of urinary nitrogen present as urea and ammonia, which are partly replaced by as yet unidentified nitrogenous substances (undetermined nitrogen); but excretion of definitely abnormal amounts of amino-acids appears to result only when the destruction of the liver cells is almost complete.

These observations support the view that in the deamination of amino-acids and the synthesis of urea the liver bears a part which cannot be entirely assumed by the rest of the body.

EXPERIMENTS ON CARBOHYDRATE METABOLISM AND DIABETES.

I. INTRAVENOUS GLUCOSE TOLERANCE OF DOGS.

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The present paper forms one of a series concerning the internal pancreatic function. Experiments dealing with quantitative relations between this function and the body mass and metabolism have already been in part presented elsewhere.[1] The series in this *Journal* will concern the nature of the pancreatic function and its rôle in metabolism. This series is fragmentary. The study was begun in the early period of the diabetic work at The Rockefeller Institute, as an attempt either to establish or disprove certain conceptions of normal and diabetic metabolism which had grown out of the previous investigation at the Harvard Medical School. Events compelled dropping this undertaking, and only a few experiments were worked in subsequently as time permitted. The portion completed may serve to clear the ground a little and reveal the scope and purpose of the original plan.

The present paper deals with the assimilation of intravenously injected glucose by dogs. Merck's anhydrous glucose was used throughout, and the concentration of solutions determined by simple weighing. Analyses for sugar in blood or plasma were performed by the original Lewis-Benedict[2] method, and those for urinary sugar by titration with Benedict's copper solution. Except when otherwise stated, the dogs were on a diet of bread and soup *ad libitum*, and experiments were begun 20 to 24 hours after the last feeding. The method of injection requires special mention.

[1] Allen, F. M., Experimental studies on diabetes, Series I, *J. Exp. Med.*, 1920, xxxi, 363, 381, 555, 575, 587; Series II, xxxi (in press).

[2] Lewis, R. C., and Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 61.

Interrupted injections were used, essentially according to the method of Blumenthal,[3] for two reasons; first, the experiments were mostly performed before Woodyatt had devised his excellent apparatus for uniform continuous infusions; second, one important purpose of the test was the comparison of the normal assimilation with that attained during exercise, and an interrupted method seemed indispensable for this purpose. Obviously, when short injections are given 15 or 20 minutes apart, there is a sudden flood of hyperglycemia, followed by a gradual decline to the time of the next injection. The excess passes rapidly into the tissues, and there is a certain basis for Blumenthal's conception of a "saturation limit" of the organism, in that it will hold a certain quantity of sugar like a sponge, and with identical dosage the assimilation is practically the same whether the injections are given continuously or at short intervals. It is true that Wilder and Sansum[4] criticized the irregularities of tolerance found in rabbits by different workers with the Blumenthal method, but there is no evidence that these irregularities were due to anything else than the failure of the earlier workers to take the precautions against fright, pain, and shock concerning which Woodyatt and his collaborators were so careful, and Woodyatt[5] recognizes that the results with his method and the Blumenthal method are essentially the same. At any rate, the plan of delivering fixed doses of glucose into a vein and allowing fixed intervals for its disposal is one valid method of testing the assimilative power. A few experiments with continuous infusion were also performed as controls.

The detailed procedure was as follows. The dogs used were sturdy, phlegmatic animals, thoroughly accustomed to the laboratory and to staying on the table without jumping down, and with all fear or nervousness lost through previous experience. The dietary tolerance of the diabetic ones was accurately known from long observations. An hour or so before the experiment, an area of the neck was shaved, if necessary, and a short portion of an external jugular vein exposed, with local anesthesia. The essential point is

[3] Blumenthal, F., *Beitr. chem. Physiol. u. Path.*, 1905, vi, 329.

[4] Wilder, R. M., and Sansum, W. D., *Arch. Int. Med.*, 1917, xix, 311.

[5] Woodyatt, R. T., *The Harvey Lectures*, 1915–16, xi, 326.

to clean a tiny portion of vein wall of every trace of fascia. The skin falls together so that the uncovered area is kept naturally moist. The dog is not tied or confined at any stage of the experiment. At the time for beginning, an assistant raises the dog's head. The operator takes a Luer syringe containing a few crystals of potassium oxalate and equipped with a needle, of No. 21 or 22 size and ⅝ inch length. He draws blood into the syringe, then detaches the needle without removing it from the vein, and immediately attaches another syringe previously filled with the solution at proper temperature and makes the injection at a convenient rate, requiring perhaps 2 to 4 minutes according to the dose. There is no bleeding when the needle is withdrawn. The dog sits or lies quietly without restraint, generally dozing, till the time of the next injection. The needle is then inserted through the same hole in the vein wall as before, the blood sample drawn, and the injection made as before. The same hole in the vein is generally used throughout the whole experiment. The blood sugar values thus obtained are a minimum, because taken at the end of the interval. A few analyses have been made immediately after injection. In some experiments blood samples were taken only hourly. The method serves satisfactorily for comparisons of assimilation, but affords no exact reckoning of relations between the sugar levels in blood and urine.

Continuous injections were given through a small cannula tied into an external saphenous vein, while blood samples were taken from the external jugular. Here also the dogs were merely watched and not confined; they mostly dozed without noticing the cannula, but were free to rise and stretch as suited their comfort. The glucose solution was merely run in from a burette fixed at a suitable height. This generally held the quantity to be given in 15 minutes, and it was one person's duty to keep the flow uniform and in particular to make sure that one-third of the quantity was delivered every 5 minutes.

With both kinds of injections, the dogs were catheterized hourly or sometimes at shorter intervals. Permanent catheterization was not used. The glucose solutions were made up in distilled water except when saline is specified.

REMARKS AND CONCLUSIONS.

1. Agreement of Results.—As shown in Table XXXII, the results in normal dogs agree satisfactorily both among themselves and with Woodyatt's findings. Giving 1.8 to 2 gm. per kilo per hour, Sansum and Woodyatt[6] found ordinarily about 2 per cent of the dose excreted, with variations between individual animals reaching as high as 10 per cent of the dose in one animal. A similar close agreement is seen here between the dogs receiving 1.5 gm. per kilo per hour. Also in harmony with Woodyatt, the dosage of 1 gm. per kilo per hour is found to be slightly above the tolerance, with the single exception shown in Table IV, Period A. Variations in the concentration have, as shown by Woodyatt, no perceptible effect upon the assimilation. Even the different modes of administration, whether continuously or in four, three, or even two injections per hour, are apparently of minor importance, though this is not certain, and it is desirable that comparisons should be based upon the same method, as has been done in this series.

2. Demonstration of Alterations of Tolerance.—(A) A reduction of tolerance, as established by Wilder and Sansum[4] in human patients, is demonstrable in a broad sense in diabetic animals. Tables XXXII and XXXIII show that in general the excretion of sugar in partially depancreatized dogs is greater than that in normal dogs, and that this excretion increases with the severity of the diabetes. Also Tables XXIX, XXX, and XXXI show increase of the sugar excretion with aggravation of the diabetes in the same animal, and the latter two were thus used[7] to demonstrate the downward progress of diabetes on excessive diets of either carbohydrate or protein even in absence of glycosuria.

(B) This general rule is subject to very marked exceptions. A mildly diabetic animal (Table XX) remained free from glycosuria with dosage of 1 gm. of glucose per kilo per hour, the tolerance thus apparently surpassing that of normal animals. The difference between Periods A and E in Table XVII evidently indicates aggravation of the diabetes during the experimental period, but even at

[6] Sansum, W. D., and Woodyatt, R. T., *J. Biol. Chem.*, 1917, xxx, 155.
[7] Allen, F. M., *J. Exp. Med.*, 1920, xxxi, 567, 569.

the close the diabetes was still very mild, so that the dog was able to live on bread without glycosuria. Yet the excretion of 37 per cent of the dose by this animal was higher than that of any of the other dogs of the series, some of which were so severely diabetic that they were kept alive only on low protein-fat diets and would have gone into a hopeless condition if this tolerance had been overstepped for only a few days. The dog in Table XXVIII had one of the severest grades of diabetes in the series, which was thoroughly controlled by diet and reduction of weight. It was proper that the test should show the benefit of such thoroughness, but not that the assimilation should appear superior to that of the two non-diabetic dogs (Tables XV and XVI) and of nearly all the mildly diabetic ones. Other comparisons between diabetic dogs in Table XXXIII show that the sugar excretion is seldom an accurate measure of the degree of diabetes as known from the food tolerance and general clinical condition. The same applies to the gradations of assimilation in conditions short of diabetes. Thus the excretion is practically identical in Tables II and XV, though one animal was normal and the other was depancreatized to an extent involving a very great lowering of tolerance as demonstrable by alimentary and subcutaneous tests.[1] The animal in Table XVI, which was very close to diabetes, excreted a bare trifle more glucose than the normal dog in Table VI and actually less than that in Table VIII. Tests of assimilation based upon the rate of excretion in comparison with the rate of intravenous injection of glucose are therefore subject to wide errors.

(C) Determinations of the concentration of sugar in the blood are necessary for the correct interpretation of such experiments.

(a) This is the only means by which the important factor of the renal function can be judged. The latter is the outstanding cause of the apparent irregularities of tolerance in these experiments. The normal dog in Table IV showed no more than a doubtful reaction in the urine when the blood sugar in Period A was found as high as 0.173 per cent. It is self-evident that these analyses taken just before injections represent minimal values. This fact has occasionally been demonstrated by taking samples, from the opposite jugular or a femoral vein as quickly as possible after finishing an injection; for example, a value of 0.457 per cent was thus

found in Table III, and one of 0.770 per cent in the diabetic dog in Table XXX, and 0.294 per cent about 2 minutes after an injection in Table XIX, Period A. This flood of sugar rapidly passes from the blood into the tissues, as already mentioned. It is obvious that the normal dog in Table IV had an exceptionally high renal threshold for sugar in Period A, especially as the temporary excess of sugar was necessarily greater with only two injections per hour; also in Period B the sugar elimination was relatively small compared with the hyperglycemia. This renal impermeability will readily explain the exceptionally high tolerance. The mildly diabetic dog in Table XVII had an exceptionally active excretory function for sugar. After discontinuance of injections in Period A, this dog had glycosuria with plasma sugar concentrations from 0.084 to 0.116 per cent; and in this and the other experiments the quantity of sugar excreted was high in comparison with the hyperglycemia. With increasing severity of diabetes, the dogs were kept on protein-fat diets, and accordingly showed uniformly high renal thresholds.[8] Thus, after discontinuance of injections, in Table XXVII there was a negative sugar reaction in the final urine sample with the plasma sugar 0.208 to 0.226 per cent. In Table XXX, Period A was performed while the dog was aglycosuric on bread diet, and there was faint glycosuria at the close with plasma sugar about 0.130 per cent; Period B was performed (November 19) after a change to protein-fat diet had become necessary on October 10, and at the close there was faint glycosuria while the plasma sugar was falling from 0.322 to 0.156 per cent, and none while it was falling from 0.156 to 0.147 per cent; Period C was performed after continuance of protein-fat diet to the following February, and in the final period there was faint glycosuria while the plasma sugar was falling from 0.294 to 0.170 per cent. Dog D4-69 (Table XXXI) was an animal with one kidney, which at different times showed plasma sugar of 0.256 to 0.264 per cent without glycosuria. Also in these animals the quantitative sugar excretion was generally low in comparison with the hyperglycemia. The writer previously[9] sug-

[8] Data will be presented in Paper II of this series. Allen, F. M., *J. Biol. Chem.*, 1920, xliii (in press).

[9] Allen, F. M., Studies concerning glycosuria and diabetes, Cambridge, 1913, 53, 54.

gested fallacies in the apparent exactness of intravenous tolerance tests, particularly the abnormal prominence of the factor of renal permeability in them. Woodyatt and his collaborators have adopted the rate of utilization as a standard, and have striven to avoid the errors of Blumenthal's "saturation limit," but their publications contain no records of tests upon nephritics or others with elevated renal thresholds.

(b) The lowered tolerance of the animals which were diabetic or near to diabetes was invariably manifest in their greater hyperglycemia. Three types of plasma sugar curves may be distinguished in the longer injections. First, the normal curve seems to be a plateau which is maintained with only accidental irregularities for as long as 10 hours, as in Table III. A greater number of experiments would have been desirable to establish this as a general rule and exclude exceptions, such as a possible falling tendency of the curve indicating improved utilization due to some reaction of the pancreas or general organism; but Woodyatt's experience that glycosuria rises for the first few hours and then maintains a constant level is opposed to any such reaction of improved utilization. Second, it was noticed[1] that at certain stages of partial pancreatectomy or diabetes there is some kind of reaction which increases the apparent tolerance for glucose given by stomach or subcutaneously, and the microscopic studies also prove that the pancreatic islands can be driven to overfunction by carbohydrate excess. Occasional animals in the proper stage of mild diabetes showed such a reaction in the intravenous tests; e.g., in Table XX a plasma sugar plateau of about 0.2 per cent was maintained over 2 hours, and then fell to 0.128 and 0.112 per cent; and in the longer experiment of Table XXX, Period A, this was more marked, for the plasma sugar which rose as high as 0.715 per cent at the close of the 2nd hour declined gradually to 0.125 per cent at the end of the 8th hour. Sometimes the reaction seems to break under longer strain, so that a transitory fall of plasma sugar concentrations is followed by a secondary rise, as in Table XV, Period A. These declines were not explainable by changes of sugar secretion. Third, this power of the pancreas remnant to react to carbohydrate excess is lost in the more severe stages of diabetes. The progress of this loss is seen in Periods B and C of Table XXX;

and the other animals with the more severe grades of diabetes show merely an excessive hyperglycemia with no tendency to decline.

(c) The combined consideration of the glycosuria and the blood sugar concentration makes the intravenous method sufficiently accurate for practical tests of tolerance, and it holds a position of special usefulness in excluding irregularities of absorption, notably in such conditions as thyroid and pituitary deficiency. Outside of such special conditions, other factors seem to predominate over any possible irregularities of absorption, and it is doubtful if the intravenous method can show the finer gradations of tolerance such as are revealed by the alimentary or subcutaneous administration of glucose. Any lowering of assimilation demonstrable by the intravenous method in this series was fully obvious in feeding tests. On the other hand, Table XVI shows a dubious outcome of the intravenous test in an animal which was very close to diabetes; and in Table XV the test is still more doubtful in an animal possessing only $\frac{1}{3}$ to $\frac{1}{4}$ of the pancreas, in which the results of alimentary or subcutaneous tests are usually plain. It is unfortunate that further tests could not be performed upon animals possessing larger fractions of pancreas. The reasons for the differences between the different modes of administration will receive fuller discussion at the close of this series of papers.

3. Water Balance.—A. Body temperature.—A slight elevation of rectal temperature was the rule in the experiments, as shown in Table IV and incidental observations in Tables XIII, XV, and XVII. The dogs were comfortable and refused offered water, with the single exception in Table XX. Any serious desiccation is therefore excluded.

B. Hydremia.—Hemoglobin estimations by the Fleischl-Miescher method were performed on three normal (Tables II, IV, and XIV), and seven partially depancreatized dogs (Tables XV to XIX, XXVII, and XXVIII). Some of the dogs were anemic from cage life and repeated bleeding experiments, but not to any degree, apparently invalidating the experimental results. A fall in hemoglobin during the injection period was the rule, sometimes with a return to the original level at the close and sometimes not. One exception was seen in the rise of hemoglobin in the diabetic dog in Table XXVIII, Period B. There was no uniform relation with the plasma

sugar level and no special difference between diabetic and non-diabetic animals. This behavior of the hemoglobin is to be expected when the blood is diluted by injection of an aqueous solution, and the determinations serve chiefly to prove that the variations in plasma sugar were not explainable by dilution or concentration of the blood. The plasma bicarbonate concentration fell during the injection period in Table XV, Period C, though the fat absorption is a possible factor here; but in Table IV, Period A, it actually rose.

C. Diuresis.—The urine was regularly less than the quantity of solution injected, and there was more or less antidiuretic action of glucose, in the sense of retention of water in the blood or in the body, in both normal and diabetic animals. On the other hand, in one diabetic animal (Table XXVII) a comparison was made between 0.9 per cent saline and 5 per cent glucose in 0.9 per cent saline. Here the hydremia was less and the urine volume greater with the glucose solution, so that in this sense glucose was a diuretic.

In relation to all the above observations, notice should be taken of one fact to avoid confusion in interpretation. Even in severe diabetes, if the tolerance has been conserved so that the greater part of a single dose of sugar is assimilated in a manner approaching the normal, the behavior in other particulars, such as diuresis, should likewise resemble the normal. The similarity of behavior of the diabetic and non-diabetic animals in this series conforms to this expectation.

4. Influence of Protein and Fat Feeding.—In connection with the question whether the endocrine pancreatic function is directly and primarily concerned with carbohydrate metabolism alone or also with protein and fat metabolism, it was desired to perform comparative assimilation tests in fasting and fed animals. Later papers in this *Journal* will corroborate existing evidence that fat feeding causes no immediate glycosuria or hyperglycemia; but in the more severe stages of diabetes protein feeding causes prompt and marked elevations of blood sugar. The greatest importance would therefore be attached to any positive results in normal and mildly diabetic animals, in which protein alone causes no hyperglycemia. In a previous experience[10] feeding tests had shown no important differences

[10] Allen, F. M., *J. Exp. Med.*, 1920, xxxi, 401.

of tolerance for starchy foods alone or with protein, but a control with intravenous glucose tests was desired to exclude possible alterations of absorption. The normal dog in Table II was given identical intravenous glucose injections, Period A fasting and Period B 5 hours after feeding 500 gm. of beef lung. The glycosuria was practically identical in the two instances, and the hyperglycemia was slightly greater in the fasting condition, especially in the early part of the test (probably a slight suggestion of "hunger glycosuria"). The partially depancreatized non-diabetic dog in Table XV was similarly tested fasting, after protein, and after fat feeding. Here also the plasma sugar reached its highest concentration of 0.208 per cent at the end of the 1st hour in the fasting period. Otherwise the plasma sugar levels did not differ appreciably and the glycosuria was practically identical. The mildly diabetic dog in Table XVII was tested first in Period A fasting; then Period B with lard feeding showed distinctly higher hyperglycemia and glycosuria; then Period C with control feeding of clay (swallowed readily in the form of soft molded balls placed in the throat) showed an equal hyperglycemia but less glycosuria; then Period D with lard feeding showed a higher hyperglycemia than before; finally Period E with protein feeding showed the highest hyperglycemia of all. Circumstances prevented a final fasting test, but the record probably indicates merely a gradual aggravation of the diabetes and cannot be regarded as proof of an influence of fat or protein feeding upon the sugar assimilation. It may be mentioned incidentally that ingestion of anything seems sometimes to raise the diabetic blood sugar, as seen in Periods B, C, and E, and the effect is not purely psychic as shown by the negative effect of tantalizing in Period E. The mildly diabetic dog in Table XIX showed no more than accidental variations in hyperglycemia and glycosuria when tested fasting or after protein or fat feeding. The greatest question was raised by the severely diabetic dog in Table XXVIII, which was known from other tests[11] to be subject to marked hyperglycemia from protein feeding alone. Here the variations in glycosuria were trivial, and the hyperglycemia was greatest in the fasting test (Period A), being slightly lower after

[11] Allen, F. M., *Am. J. Med. Sc.*, 1917, cliii, 363, 365.

protein and markedly lower after fat feeding. The vomiting of undigested lung by this dog after the close of the injections, of fat by the dog in Table XVII, Period D, and of clear water in the fasting period of Table XIV, raises the question whether intravenous glucose injections interfere with alimentary absorption. There was marked milkiness of the plasma in Table XV, Period C, but little or none in the other fat feeding experiments. The experiments at least afford no evidence of a direct participation of the pancreatic hormone in protein or fat metabolism, but their decisiveness is open to question.

TABLE I.

Dog F6–18.

Weight 11 kg. Intravenous glucose injections, 1 gm. per kg. per hr. in 10 per cent solution (3 injections per hr.).

Date.	Time.	Urine.		Plasma sugar.	Remarks.
		Vol- ume.	Glu- cose.		
1917		*cc.*	*per cent*	*per cent*	
Dec. 20	3.25 p.m.	27	0	0.086	3.50 p.m. started injections.
	4.45 "	18	0.56	0.109	
	5.45 "	8	0.77	0.118	5.35 p.m. finished injections.
	7.10 "	56	0	0.112	
	8.10 "	40	0	0.109	Total solution injected... 220 cc.
					Total glucose injected.... 22 gm.
					Total urine excreted..... 122 cc.
					Total glucose excreted.... 0.16 gm.
					Per cent of dose........ 0.73 per cent

TABLE II.

Dog C3-93.

Weight 15.5 kg. Normal. Intravenous glucose injections, 1 gm. per kg. per hr. in 20 per cent solution (3 injections per hr.).

Date.	Time.	Urine.				Plasma sugar.		Hemoglobin.		Remarks.
		Volume.		Glucose.						
		A	B	A	B	A	B	A	B	
		cc.	*cc.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
1916										
May 1	9.00 a.m.						0.117		105	Fed 500 gm. lung.
Period A.	11.00 "						0.114		98	Started injections.
May 31	2.00 p.m.					0.133	0.111	105	100	A = Fasting.
Period B.	2.20 "	5	19	0	0	0.153	0.112	92	87	B = After feeding 500 gm. lung.
	2.40 "	8	18	2.08	0.29	0.132	0.116	85		
	3.00 "	8	20	1.64	0.82	0.095	0.123	89		
	3.20 "	4	13	1.05	0.80	0.119	0.084	98	100	
	3.40 "	5	45	1.72	0.97	0.103	0.080	104	100	
	4.00 "	6	70	1.52	Faint.	0.098	0.099	106	102	
	4.20 "	13	12	1.39	"	0.092	0.128	110	98	
	4.40 "		31	0.80	0.54	0.143	0.114	111	100	
	5.00 "	4	15	0.74	0.75	0.161	0.118	105	100	Finished injections.
	5.30 "	18		0	0.68	0.105	0.093	104	95	
	7.00 "	58	190	0	Faint.	0.104	0.095	105		

Periods A and B.
Total solution injected.................. 155 cc.
  "   glucose   "   .... 31 gm.
Period A.
Total urine excreted.................. 129 cc.
  "   glucose   "   .... 0.67 gm.
Per cent of dose.................. 2.16 per cent.

Period B.
Total urine excreted.................. 433 cc.
  "   glucose   "   .... 0.89 gm.
Per cent of dose.................. 2.87 per cent.

TABLE III.

Dog F6-03.

Weight 20 kg. Intravenous glucose injections, 1 gm. per kg. per hr. in 20 per cent solution (3 injections per hr.).

Date.	Time.	Urine.		Plasma sugar.	Remarks.
		Volume.	Glucose.		
1917		*cc.*	*per cent*	*per cent*	
Nov. 19	10.45 a.m.	70	0	0.119	11.10 a.m. started injections.
	12.05 p.m.	12	2.06	0.149	
	1.05 "	4	2.12	0.185	
	2.05 "	21	2.38	0.172	Plasma sugar immediately following
	3.05 "	15	0.76	0.147	injection 0.457 per cent.
	4.05 "	124	0.35	0.147	
	5.05 "	65	0.52	0.159	
	6.05 "	77	0.47	0.200	
	7.05 "	92	0.38	0.175	
	8.05 "	86	0.41	0.164	
	9.05 "	78	0.23	0.196	8.55 p.m. finished injections.
	10.00 "	35	Faint.	0.113	
	11.00 "	39	0	0.110	Total solution injected.......... 1,000 cc.
					Total glucose injected.......... 200 gm.
					Total urine excreted.......... 648 cc.
					Total glucose excreted.......... 2.95 gm.
					Per cent of dose... 1.48 per cent.

TABLE IV.

Dog C3-47.

Weight 15 kg. Normal. Comparative intravenous glucose injections of 1 and 1.5 gm. per kg. per hr. (2 injections per hr.).

Date.	Time.	Urine.				Plasma sugar.		CO_3		Hemoglobin.		Temperature.		Remarks.
		Volume.		Glucose.										
		A	B	A	B	A	B	A	B	A	B	A	B	
		cc.	cc.	per cent	per cent	per cent	per cent	vol. per cent	vol. per cent	per cent	per cent	°F.	°F.	
1916														
Jan. 21	1.30 p.m.	150	171	0	0	0.120	0.133	50		121	100	101.8	101.4	1.40 p.m. started injections.
Period A.	2.00 "	25	40	(?)	1.56	0.173	0.232	55.7		82	80	102	101.6	
	2.30 "	69	145	0	0.32	0.136	0.356	54.8		81	83	102	101.6	
Jan. 27	3.00 "	119	245	0	Very faint.	0.154	0.130*	55.7		79	103*	102.2	102.1	3.06 p.m. finished injections.
Period B.	3.30 "	176	50	0	0.37	0.125	0.196	59.5		84	88	102	102	
	3.40 "	110	95	0	0	0.114	0.128	59.5		84	87	101.6	101.8	

Period A. 1.0 gm. per kg. per hr. in 5 per cent solution.
Total solution injected................. 600 cc.
  " glucose   " .... 30 gm.
  " urine excreted............. 499 cc.
  " glucose   " .... 0 gm.
Per cent of dose............. 0 per cent.

Period B. 1.5 gm. per kg. per hr. in 7.5 per cent solution.
Total solution injected................. 600 cc.
  " glucose   " .... 45 gm.
  " urine excreted............. 575 cc.
  " glucose   " .... 1.02 gm.
Per cent of dose............. 2.27 per cent.

* This simultaneous break in the sugar and hemoglobin curves is unexplained.

TABLE V.

Dog F6-33.

Weight 12 kg.   Intravenous glucose injections, 1.5 gm. per kg. per hr. in 20 per cent solution (3 injections per hr.).

Date.	Time.	Urine.		Plasma sugar.	Remarks.
		Volume.	Glucose.		
1917		*cc.*	*per cent*	*per cent*	
May 22	4.40 p.m.	20	0	0.093	Started injections.
Period A.	5.40 "	27	1.93	0.088	
	6.40 "	22	1.37	0.085	6.20 p.m. finished injections.
	7.40 "	32	Faint.	0.115	
	8.40 "	15	"	0.114	Total solution injected............. 180 cc.
					Total glucose injected 36 gm.
					Total urine excreted.. 86 cc.
					Total glucose excreted 0.82 gm.
					Per cent of dose...... 2.28 per cent.
June 13	4.30 p.m.	41	0	0.111	Started injections.
Period B.	5.30 "	14	2.94	0.170	
	6.30 "	12	2.93	0.132	
	7.30 "	14	Faint.	0.128	7.10 finished injections.
	8.30 "	56	0	0.110	
					Total solution injected 270 cc.
					Total glucose injected. 54 gm.
					Total urine excreted.. 96 cc.
					Total glucose excreted 0.76 gm.
					Per cent of dose...... 1.41 per cent.

TABLE VI.

Dog F6-34.

Weight 7.6 kg. Intravenous glucose injections, 1.5 gm. per kg. per hr. (3 injections per hr.).

Date.	Time.	Urine.		Plasma sugar.	Remarks.
		Vol-ume.	Glucose.		
1917		*cc.*	*per cent*	*per cent*	
June 12	4.30 p.m.	25	0	0.149	Started injections.
	5.30 "	14	3.23	0.208	
	6.30 "	17	3.22	0.176	6.10 p.m. finished injections.
	7.30 "	32	Faint.	0.102	
	8.30 "	20	0	0.103	Total solution injected 114 cc.
					Total glucose injected. 22.8 gm.
					Total urine excreted... 83 cc.
					Total glucose excreted. 1.0 gm.
					Per cent of dose...... 4.40 per cent.

TABLE VII.

Dog F6-83.

Weight 9 kg. Intravenous glucose injections, 1.5 gm. per kg. per hr. (3 injections per hr.).

Date.	Time.	Urine.		Plasma sugar.	Remarks.
		Vol-ume.	Glucose.		
1917		*cc.*	*per cent*	*per cent*	
May 23	4.20 p.m.	25	0	0.109	5.40 p.m. started injections.
	6.40 "	52	3.70	0.159	
	7.40 "	58	2.86	0.152	
	8.40 "	124	2.33	0.218	8.20 p.m. finished injections.
	9.40 "	38	Faint.	0.102	
	10.40 "	12	Very faint.	0.114	Total solution injected 203 cc.
					Total glucose injected 40.5 gm.
					Total urine excreted.. 284 cc.
					Total glucose excreted 6.47 gm.
					Per cent of dose...... 1.60 per cent.

TABLE VIII.

Dog F6-84.

Weight 9 kg. Intravenous glucose injections, 1 gm. per kg. per hr. (3 injections per hr.).

Date.	Time.	Urine.		Plasma sugar.	Remarks.
		Vol-ume.	Glucose.		
1917		*cc.*	*per cent*	*per cent*	
May 23	4.35 p.m.	18	0	0.086	5.15 p.m. started injections.
	6.15 "	50	2.63	0.175	
	7.15 "	26	2.80	0.179	
	8.15 "	90	1.50	0.104	7.55 p.m. finished injections.
	9.15 "	63	Faint.	0.075	
	10.15 "	25	Very faint.	0.089	Total solution injected 203 cc.
					Total glucose injected 40.5 gm.
					Total urine excreted.. 244 cc.
					Total glucose excreted 3.41 gm.
					Per cent of dose...... 8.40 per cent.

TABLE IX.

Dog G7-39.

Weight 11 kg. Intravenous glucose injections, 1.5 gm. per kg. per hr. in 20 per cent solution (3 injections per hr.).

Date.	Time.	Urine.		Plasma sugar.	Remarks.
		Vol-ume.	Glucose.		
1918		*cc.*	*per cent*	*per cent*	
July 10	1.50 p.m.	15	0	0.118	Started injections.
	2.50 "	10	2.00	0.167	
	3.50 "	5	2.80	0.137	
	4.50 "	148	0.26	0.232	4.35 p.m. finished injections.
	5.50 "	44	Very faint.	0.085	
	6.50 "	66	0	0.131	Total solution injected 248 cc.
					Total glucose injected. 49.5 gm.
					Total urine excreted... 273 cc.
					Total glucose excreted. 0.73 gm.
					Per cent of dose....... 1.50 per cent.

TABLE X.
Dog F6-31.

Weight 18 kg.   Intravenous glucose injections, 1.5 gm. per kg. per hr. in 20 per cent solution (3 injections per hr.).

Date.	Time.	Urine.		Plasma sugar.	Remarks.
		Vol-ume.	Glucose.		
1918		*cc.*	*per cent*	*per cent*	
April 5	2.30 p.m.	58	0	0.135	Started injections.
	3.30 "	62	0.66	0.182	
	4.30 "	106	0.22	0.132	
	5.30 "	80	0.28	0.192	5.10 p.m. finished injections.
	6.30 "	50	0.59	0.152	
	7.30 "	50	0.12	0.106	Total solution injected 405 cc.
					Total glucose injected. 81 gm.
					Total urine excreted... 348 cc.
					Total glucose excreted. 1.22 gm.
					Per cent of dose...... 1.50 per cent.

TABLE XI.
Dog G7-69.

Weight 8 kg.   Intravenous glucose injections, 1.5 gm. per kg. per hr. in 20 per cent solution (3 injections per hr.).

Date.	Time.	Urine.		Plasma sugar.	Remarks.
		Vol-ume.	Glucose.		
1918		*cc.*	*per cent*	*per cent*	
July 10	1.55 p.m.	14	0	0.122	Started injections.
	2.55 "	23	0.65	0.122	
	3.55 "	33	0.48	0.164	
	4.55 "	60	0.23	0.156	4.35 p.m. finished injections.
	5.55 "	56	Very faint.	0.147	
					Total solution injected 180 cc.
	6.55 "	20	0	0.102	Total glucose injected. 36 gm.
					Total urine excreted... 192 cc.
					Total glucose excreted. 0.45 gm.
					Per cent of dose...... 1.28 per cent.

TABLE XII.

Dog F6-32.

Weight 14 kg. Intravenous glucose injections, 1.5 gm. per kg. per hr. in 20 per cent solution (3 injections per hr.).

Date.	Time.	Urine.		Plasma sugar.	Remarks.
		Vol-ume.	Glu-cose.		
1918		*cc.*	*per cent*	*per cent*	
April 5	2.20 p.m.	72	0	0.120	Started injections.
	3.20 "	46	0.48	0.204	
	4.20 "	47	0.28	0.192	
	5.20 "	132	0.40	0.208	
	6.20 "	70	0.28	0.250	6.05 p.m. finished injections.
	7.20 "	38	0.23	0.109	
					Total solution injected... 420 cc.
					Total glucose injected.... 84 gm.
					Total urine excreted..... 333 cc.
					Total glucose excreted... 1.17 gm.
					Per cent of dose......... 1.40 per cent.

TABLE XIII.

Dog F6-05.

Weight 15 kg. Intravenous glucose injections, 1.5 gm. per kg. per hr. in 10 per cent solution (4 injections per hr.).

Date.	Time.	Urine.		Plasma sugar.	Remarks.
		Vol-ume.	Glucose.		
1917		*cc.*	*per cent*	*per cent*	
Dec. 4	10.00 a.m.	50	0	0.109	Started injections.
	11.00 "	148	Faint.	0.118	
	12.00 "	256	Slight.	0.123	
	1.00 p.m.	360	0.18	0.143	
	2.00 "	180	0.78	0.170	
	3.00 "	132	0.32	0.151	3.20 p.m. temperature 39.6°C.
	4.00 "	330	0.14	0.104	
	5.00 "	146	0.19	0.104	6.30 p.m. temperature 39.1°C.
	6.00 "	225	0.25	0.105	6.40 p.m. finished injections.
	7.00 "	146	0.15	0.119	
	8.00 "	40	Very. faint.	0.109	Total solution in-jected.......... 2,027 cc.
					Total glucose injected 202.7 gm.
					Total urine excreted. 1,963 cc.
					Total glucose ex-creted........... 3.68 gm.
					Per cent of dose.... 1.88 per cent.

TABLE XIV.

Dog C3-92.

Weight 18 kg. Normal. Continuous intravenous glucose injection, 2 gm. per kg. per hr. in 5 per cent solution.

Date.	Time.	Urine.		Plasma sugar.	Hemoglobin.	Remarks.
		Vol-ume.	Glucose.			
1916		*cc.*	*per cent*	*per cent*	*per cent*	
Aug. 4	2.25 p.m.	20	0	0.114	90	2.30 p.m. started injection.
	3.30 "	85	3.00		77	Vomited during experiment 300 cc. of
	4.30 "	90	2.50	0.330	78	clear fluid, sugar-free.
	5.30 "	320	0.50	0.300	77	
	6.30 "	200	Faint.			Finished injection.
	7.30 "	135	0	0.111		
						Total solution in-jected.......... 2,160 cc.
						Total glucose in-jected.......... 108 gm.
						Total urine ex-creted.......... 850 cc.
						Total glucose ex-creted.......... 6.4 gm.
						Per cent of dose... 5.90 per cent.

TABLE XV.

Dog C3-88.

Weight 10 kg. Non-diabetic; ¼ to ⅓ of pancreas present.* Intravenous glucose injections, 1 gm. per kg. per hr. in 20 per cent solution (3 injections per hr.).

Date.	Time.	Urine. Volume.	Urine. Glucose.	Plasma sugar.	Hemoglobin.	CO₂	Remarks.
1916		*cc.*	*per cent*	*per cent*	*per cent*	*vol. per cent*	
May 8 Period A.	2.00 p.m.		0	0.117	90		2.02 p.m. injections started.
	2.20 "	4	1.14	0.170	83		
	2.40 "	3	1.82	0.160	90		
	3.00 "	4	2.32	0.208	88		
	3.20 "			0.123	84		
	3.40 "	8	1.85	0.105	86		Low renal threshold.
	4.00 "			0.147			Temperature 102.9°F.
	4.20 "	8	1.47	0.143	82		Note secondary rise of plasma sugar.
	4.40 "	9	1.93	0.164	86		4.42 p.m. finished injections.
	5.00 "	19	0.22	0.122			Temperature 102°F.
	5.30 "	22	Faint.	0.116	90		
	6.30 "	24	Doubtful.				Total solution injected.......... 150 cc.
	7.15 "	17	0	0.102	87		Total glucose injected.......... 30 gm. Total urine excreted.......... 118 cc. Total glucose excreted.......... 0.66 gm. Per cent of dose... 2.20 per cent.
June 2 Period B.	2.05 p.m.		0	0.133	95		8.00 a.m. fed 500 gm. of lung. 2.05 p.m. started injections.
	2.20 "	16	0.51	0.164	74		
	2.40 "			0.164	76		
	3.00 "	18	0.94	0.149	74		
	3.20 "	8	1.10	0.145	85		
	3.40 "	5	1.38	0.167	87		
	4.00 "	15	1.06	0.132	85		
	4.20 "	4	1.54	0.167	90		
	4.40 "	5	0.88	0.164			4.42 p.m. finished injections.
	5.00 "	15	0.23	0.102			
	5.30 "	92	0	0.087			Total solution injected.......... 150 cc.
	6.30 "	21	0				Total glucose injected.......... 30 gm.
	7.15 "	43	0	0.118			Total urine excreted.......... 238 cc.† Total glucose excreted.......... 0.60 gm. Per cent of dose... 2.0 per cent.

* All operations were performed under ether anesthesia.

† The surplus water excreted is presumably derived from the food.

TABLE XV—*Concluded.*

Date.	Time.	Urine.		Plasma sugar.	Hemoglobin.	CO_2	Remarks.
		Vol. ume.	Glucose.				
1916		*cc.*	*per cent*	*per cent*	*per cent*	*vol. per cent*	
May 25	3.00 p.m.		0	0.102	90	55.8	9.30 a.m. fed 200 gm. of lard.
Period	3.20 "	4	Faint.	0.161	88	54.8	3.00 p.m. started injections.
C.	3.40 "	6	0.76	0.147	85	50.4	Heavy lipemia.
	4.00 "	3	0.54	0.156	70	54.8	Moderate "
	4.20 "	4	1.24	0.161	65	50.4	
	4.40 "			0.189	74	56.2	Finished injections.
	5.00 "	3	1.37	0.185	74	56.2	
	5.20 "			0.128	65	51.4	Heavy lipemia.
	5.40 "	15	0.26	0.143	62	56.2	" "
	6.00 "	2	1.33	0.143	70	56.2	
	6.30 "	15	Faint.	0.083	75	56.2	Total solution in-
	7.30 "	5	Very faint.				jected.......... 90 cc.
							Total glucose in-
	8.15 "	13	0	0.100	71	55.8	jected.......... 20 gm.
							Total urine ex-
							creted.......... 70 cc.
							Total glucose ex-
							creted.......... 0.34 gm.
							Per cent of dose... 1.70 per cent.

TABLE XVI.

Dog B2-00.

Weight 14 kg. Partially depancreatized non-diabetic. Two subsequent operations, removing first 0.8 gm. and then 0.1 gm. of pancreas, were required to produce diabetes. Intravenous glucose injections, 1.5 gm. per kg. per hr. in 5 per cent solution (3 injections per hr.).

Date.	Time.	Urine.		Plasma sugar.	Hæmoglobin.	Remarks.
		Volume.	Glucose.			
1916		*cc.*	*per cent*	*per cent*	*per cent*	
Jan. 31	1.30 p.m.		0	0.105	1.12	1.45 p.m. started injections.
	2.00 "	60	0.36			
	2.20 "	90	0.95	0.288	98	
	2.40 "	100	0.50	0.145	91	3.00 p.m. finished injections.
	3.30 "	175	0	0.091	93	
	3.45 "	25	0	0.088	85	Total solution injected. 560 cc.
	4.00 "	30	0	0.098	80	
						Total glucose injected. 28 gm.
						Total urine excreted... 480 cc.
						Total glucose excreted. 1.57 gm.
						Per cent of dose...... 5.60 per cent.

TABLE XVII.

Dog B2-01.

Weight 14 kg. Partially depancreatized. Mildly diabetic. Continuous intravenous glucose injections, 1.2 gm. per kg. per hr. in 5 per cent solution.

Date.	Time.	Urine.		Plasma sugar.	Hemoglo- bin.	Remarks.
		Vol- ume.	Glucose.			
1916		*cc.*	*per cent*	*per cent*	*per cent*	
May 17	2.25 p.m.	25	0	0.116	107	2.30 p.m. started injections.
Period	3.00 "	76	2.44	0.228	103	
A.	4.30 "	190	1.47	0.286	95	Temperature 39.2°C.
	5.00 "	160	1.21	0.238	83	
	5.30 "	95	1.06	0.222	90	Finished injections.
	6.30 "	31	0.34	0.084	99	Note low renal threshold.
	7.10 "	19	Slight faint.	0.087	85	
	8.30 "	69		0.116	90	Total solution in- jected......... 1,008 cc. Total glucose in- jected......... 50.4 gm. Total urine ex- creted......... 640 cc. Total glucose ex- creted......... 7.64 gm. Per cent of dose... 14.1 per cent.
May 24	8.25 a.m.	50	0	0.087	108	Fed 200 gm. of lard.
Period	2.25 p.m.	2	0	0.122	110	3.00 p.m. started injections.
B.	4.00 "	110	1.89	0.385	102	
	5.00 "	216	2.13	0.435	92	
	6.00 "	350	2.04	0.358	97	Finished injections.
	7.00 "	63	1.73	0.182	98	
	8.00 "	10	0.45	0.147	98	Total solution in- jected......... 1,008 cc. Total glucose in- jected......... 50.4 gm. Total urine ex- creted......... 758 cc. Total glucose ex- creted......... 14.97 gm. Per cent of dose... 27.7 per cent.
	9.30 "	7	Faint.	0.147	98	
May 29	8.45 a.m.	20	0	0.102	95	Fed 300 gm. of white clay.
Period	9.45 "			0.120	96	
C.	2.00 p.m.	2	0	0.103	97	2.30 p.m. started injections.
	3.30 "	74	2.04	0.345	74	
	4.30 "	320	2.38	0.500	66	

TABLE XVII—*Continued*.

Date.	Time.	Urine.		Plasma sugar.	Hemoglobin.	Remarks.
		Volume.	Glucose.			
1916		*cc.*	*per cent*	*per cent*	*per cent*	
May 29	5.30 p.m.	278	3.70	0.385	68	Finished injection.
Period	6.30 "	30	2.22	0.182		Drank 250 cc. of water.
C.—Con-	7.45 "			0.179		" 200 " " "
tinued.	9.00 "	16	0.28	0.135	84	
						Total solution injected.......... 1,008 cc.
						Total glucose injected.......... 50.4 gm.
						Total urine excreted.......... 710 cc.
						Total glucose excreted.......... 9.84 gm.
						Per cent of dose... 18.2 per cent.
June 1	2.40 p.m.			0.104	92	8.00 a.m. fed 200 gm. of lard.
Period	4.00 "	30	3.65	0.357	88	3.00 p.m. started injections. Slight
D.						lipemia.
	5.00 "	348	2.38	0.371	75	Negative lipemia.
	6.00 "	182	2.64	0.417	77	Finished injections. Negative lipe-
	7.00 "	40	4.17	0.193	88	mia. Vomited about 25 gm. of
	8.00 "	22	Faint.	0.145		lard. Drank 200 cc. of water.
	9.30 "	65	0	0.119	77	Total solution injected.......... 1,008 cc.
						Total glucose injected.......... 50.4 gm.
						Total urine excreted.......... 687 cc.
						Total glucose excreted.......... 15.86 gm.
						Per cent of dose... 31.5 per cent.
June 5	9.35 a.m.		0	0.093	85	Tantalized with meat for 5 min., not
Period						allowed to eat it.
E.	9.40 "			0.093	87	Fed 500 gm. of lung.
	9.50 "			0.109	82	
	10.10 "			0.119	80	
	10.25 "		0	0.156	78	3.20 p.m. started injections.
	4.20 p.m.	123	3.58	0.358	70	
	5.20 "	232	3.85	0.477	73	
	6.20 "	240	2.18	0.264	72	Finished injections.

TABLE XVII—*Concluded*.

Date.	Time.	Urine.		Plasma sugar.	Hemoglo- bin.	Remarks.
		Vol- ume.	Glucose.			
1916		*cc.*	*per cent*	*per cent*	*per cent*	
June 5	7.20 p.m.	14	0.32	0.122	74	
Period	8.20 "	19	Faint.	0.182	71	Total solution in-
E.—	9.50 "	19	"	0.121		jected.......... 1,008 cc.
Con-						Total glucose in-
tinued						jected...,...... 50.4 gm.
						Total urine excreted 647 cc.
						Total glucose ex-
						creted.......... 18.61 gm.
						Per cent of dose... 37.0 per cent.

TABLE XVIII.

Dog B2-02.

Weight 10.5 kg. Partially depancreatized. Intravenous glucose injections, 1.5 gm. per kg. per hr. in 5 per cent solution in 0.9 per cent saline (3 injections per hr.).

Date.	Time.	Urine.		Plasma sugar.	Hemoglo- bin.	Remarks.
		Vol- ume.	Glucose.			
1916		*cc.*	*per cent*	*per cent*	*per cent*	
Feb. 2	1.55 p.m.		0	0.111	114	2.15 p.m. started injections.
	2.30 "	10	2.32	0.209	108	
	2.50 "	25	2.38	0.271	106	
	3.10 "	35	2.13	0.250	97	2.55 p.m. finished injections.
	3.25 "	12	0.64	0.200	104	
	3.40 "	10	Faint.	0.137	103	Total solution injected 316 cc.
	3.55 "	10	0	0.125	99	Total glucose injected 15.8 gm.
						Total urine excreted.. 102 cc.
						Total glucose excreted 1.66 gm.
						Per cent of dose...... 10.5 per cent.

Dog C3-74.

Weight 18 kg. Partially depancreatized. Mildly diabetic. Intravenous glucose injections, 1 gm. per kg. per hr. in 20 per cent solution (3 injections per hr.).

Date.	Time.	Urine. Vol- ume.	Glucose.	Plasma sugar.	Hemoglo- bin.	Remarks.
1916		*cc.*	*per cent*	*per cent*	*per cent*	
May 8	3.00 p.m.			0.119	88	Started injections.
Period	3.20 "	8	1.82	0.173	91	3.05 p.m. blood taken 2 minutes after
A.	3.40 "	6	3.60	0.263	91	finishing injection; plasma sugar
	4.00 "	19	1.22	0.286	90	0.294 per cent.
	4.20 "	10	0.86	0.303	94	
	4.40 "	23	0.82	0.312	92	Finished injections.
	5.00 "	22	0.22	0.238	94	
	5.40 "	20	0.31			Total solution in-
	6.20 "			0.137	96	jected.......... 180 cc.
	6.40 "	20	Faint.			Total glucose in-
	7.00 "			0.126	92	jected.......... 36 gm.
	7.40 "	20	Very			Total urine excreted 174 cc.
			faint.			Total glucose ex-
	8.20 "	20	Very	0.125	93	creted.......... 0.99 gm.
			faint.			Per cent of dose.... 3.02 per cent.
May 23	2.10 "		0	0.139	88	10.25 a.m. fed 1,800 gm. of lung.
Period	2.45 "	20	0.83	0.227	87	
B.	3.05 "	19	0.95	0.250	87	2.25 p.m. started injections.
	3.25 "	15	1.54	0.264	84	
	3.45 "	18	2.08	0.286	85	
	4.05 "	18	2.38	0.286		Finished injections.
	4.20 "	21	1.93	0.313	86	
	5.00 "	19	0			Total solution in-
	5.40 "			0.185	88	jected.......... 180 cc.
	6.00 "	49	0			Total glucose in-
	6.20 "			0.139	85	jected.......... 36 gm.
	7.10 "	53	0			Total urine excreted 288 cc.
	7.40 "	56	0	0.137	82	Total glucose ex-
						creted.......... 1.79 gm.
						Per cent of dose.... 4.98 per cent.
May 12	2.00 "		0	0.097	90	10.00 a.m. fed 200 gm. of lard.
Period	2.20 "	13	0.65	0.151	95	2.00 p.m. started injections.
C.	2.40 "	15	0.70	0.232	85	
	3.00 "	15	0.66	0.162	88	
	3.20 "	18	0.49	0.167	85	
	3.40 "	14	0.61	0.137	88	Finished injections.
	4.00 "	12	0.65	0.107	85	
	4.20 "	10	0			Total solution in-
	4.40 "	11	0			jected.......... 180 cc.
	5.20 "	10	0	0.099	83	Total glucose in-
	6.20 "	60	0	0.111	78	jected.......... 36 gm.
	7.20 "	190	0	0.115	89	Total urine excreted 368 cc.
						Total glucose ex-
						creted.......... 0.54 gm.
						Per cent of dose.... 1.50 per cent.

TABLE XX.

Dog B2-88.

Weight 13 kg.   Mild diabetes.   Glycosuria absent on protein-fat diets with addition of 100 gm. of bread; present with addition of bread to 200 gm.   Intravenous glucose injections, 1 gm. per kg. per hr. in 5 per cent solution (3 injections per hr.).

Date.	Time.	Urine.		Plasma sugar.	Remarks.
		Vol-ume.	Glu-cose.		
1915		*cc.*	*per cent*	*per cent*	
Dec. 2	1.20 p.m.		0	0.118	11.40 a.m. drank 500 cc. of water, also at 1.20 p.m.
	1.59 "	120	0	0.200	1.44 p.m. started injections.
	2.20 "	64	0	0.200	
	2.43 "	73	0	0.232	
	3.05 "	100	0	0.192	
	3.38 "	106	0	0.200	
	3.54 "	64	0	0.200	
	4.15 "	90	0	0.200	
	4.37 "	90	0	0.128	
	4.58 "	110	0	0.112	5.08 p.m. finished injections.
	5.24 "	117	0	0.121	
					Total solution injected... 860 cc.
					Total glucose injected.... 43.3 gm.
					Total urine excreted..... 934 cc.
					Total glucose excreted.... 0 gm.
					Per cent of dose......... 0 per cent.

TABLE XXI.

Dog E5-97.

Weight 14 kg. Mild diabetes. Glycosuria absent on diet of beef lung with 100 gm. of bread; present with addition of bread to 200 gm. Intravenous glucose injections, 1 gm. per kg. per hr. in 20 per cent solution (3 injections per hr.).

| Date. | Time. | Urine. | | Plasma sugar. | Remarks. |
		Volume.	Glu-cose.		
1917		*cc.*	*per cent*	*per cent*	
Nov. 1	4.05 p.m.		0	0.095	4.20 p.m. started injections.
	5.20 "	No urine.		0.286	
	6.25 "	70	2.44	0.294	6.10 p.m. finished injections.
	7.30 "	No urine.		0.164	
	8.30 "	40	1.47	0.145	Total solution injected.............. 140 cc.
					Total glucose injected.. 28 gm.
					Total urine excreted... 110 cc.
					Total glucose excreted. 2.30 gm.
					Per cent of dose....... 8.2 per cent.

TABLE XXII.

Dog F6-00.

Weight 20 kg. Mild diabetes. Glycosuria absent on diet of 1 kg. of lung and 100 gm. of bread; heavy on increasing bread to 200 gm. Intravenous glucose injections, 1 gm. per kg. per hr. in 20 per cent solution (3 injections per hr.).

| Date. | Time. | Urine. | | Plasma sugar. | Remarks. |
		Vol-ume.	Glu-cose.		
1918		*cc.*	*per cent*	*per cent*	
Feb. 7	2.15 p.m.		0	0.127	2.25 p.m. started injections.
	3.20 "	25	3.64	0.555	
	4.20 "	87	4.98	0.555	
	5.20 "	180	5.89	0.667	5.10 p.m. finished injections.
					Total solution injected.... 300 cc.
					Total glucose injected..... 60 gm.
					Total urine excreted...... 292 cc.
					Total glucose excreted..... 15.84 gm.
					Per cent of dose.......... 26.4 per cent.

TABLE XXIII.

Dog E5-95.

Weight 16 kg. Moderate diabetes. Glycosuria absent on diet of 80 gm. of lung and 100 gm. of suet; present with addition of 50 gm. of bread. Intravenous glucose injections, 1 gm. per kg. per hr. in 20 per cent solution (4 injections per hr.).

Date.	Time.	Urine.		Plasma sugar.	Remarks.
		Vol-ume.	Glu-cose.		
1917		*cc.*	*per cent*	*per cent*	
Nov. 1	1.20 p.m.		0	0.109	1.40 p.m. started injections.
	2.35 "	22	3.62	0.357	
	3.40 "	90	4.31	0.416	3.25 p.m. finished injections.
	4.40 "	32	5.21	0.200	
	5.40 "	10	1.87	0.218	Total solution injected.... 160 cc.
					Total glucose injected..... 32 gm.
					Total urine excreted...... 154 cc.
					Total glucose excreted..... 6.55 gm.
					Per cent of dose.......... 20.4 per cent.

TABLE XXIV.

Dog D4-62.

Weight 19 kg. Moderate diabetes. Glycosuria absent on diet of 1 kg. of lung; heavy with the addition of 50 gm. of bread. Intravenous glucose injections, 1 gm. per kg. per hr. in 20 per cent solution (3 injections per hr.).

Date.	Time.	Urine.		Plasma sugar.	Remarks.
		Vol-ume.	Glucose.		
1916		*cc.*	*per cent*	*per cent*	
Jan. 31	1.45 p.m.		0	0.100	1.50 p.m. started injections.
	2.05 "	10	2.94	0.196	
	2.25 "	21	3.23	0.385	
	2.45 "	25	4.35	0.385	
	3.05 "	21	5.56	0.435	
	3.25 "	28	6.25	0.455	
	3.45 "	27	5.41	0.455	3.50 p.m. finished injections.
	4.05 "	33	4.54	0.416	
	5.05. "	21	3.57	0.384	Total solution injected.. 223 cc.
	6.05 "	4	2.08	0.286	Total glucose injected... 44.3 gm.
	7.30 "	7	Faint.	0.137	Total urine excreted.... 207 cc.
	10.00 "	10	0	0.088	Total glucose excreted... 8.77 gm.
					Per cent of dose........ 19.8 per cent.

TABLE XXV.

Dog D4-84.

Weight 14 kg. Moderate diabetes. Slight glycosuria on 1 kg. of beef lung. Intravenous glucose injections, 1 gm. per kg. per hr. in 10 per cent solution (4 injections per hr.).

| Date. | Time. | Urine. | | Plasma sugar. | Remarks. |
		Vol-ume.	Glu-cose.		
1917		*cc.*	*per cent*	*per cent*	
Oct. 31	1.30 p.m.			0.154	Started injections.
	2.30 "			0.400	
	3.30 "			0.525	Finished injections.
	4.30 "			0.370	
	5.40 "			0.200	Total solution injected.... 280 cc.
					Total glucose injected..... 28 gm.
					Total urine, in 24 hrs., 390 cc. with 1.6 per cent glucose.
					Total glucose excreted..... 6.24 gm.
					Per cent of dose.......... 22.2 per cent.

TABLE XXVI.

Dog E5-19.

Weight 9.0 kg. Severe diabetes. Glycosuria absent on diet of 100 gm. of lung and 100 gm. of suet; faint on 200 gm. of lung and 100 gm. of suet; heavy on 300 gm. of lung and 100 gm. of suet. Intravenous glucose injections, 1 gm. per kg. per hr. in 5 per cent solution in 0.9 per cent saline (4 injections per hr.)

| Date. | Time. | Urine. | | Plasma sugar. | Remarks. |
		Volume.	Glu-cose.		
1917		*cc.*	*per cent*	*per cent*	
Oct. 31	10.15 a.m.		0	0.133	Started injections.
	11.15 "	Not cath-eterized.		0.322	
	12.15 p.m.	57	5.10	0.475	Finished injections.
	1.15 "	15	3.29	0.278	
	2.15 "	13	2.16	0.286	Total solution injected. 420 cc.
					Total glucose injected.. 18 gm.
					Total urine excreted... 85 cc.
					Total glucose excreted.. 3.7 gm.
					Per cent of dose....... 20.5 per cent.
					Glucose in 85 cc. of additional urine up to next morning too little to titrate.

TABLE XXVII.

Dog B2-70.

Weight 14 kg. Partially depancreatized. Severe diabetes. Tolerance 500 gm. of lung. Period A, intravenous glucose injections, 1.5 gm. per kg. per hr. in 5 per cent solution in 0.9 per cent saline (3 injections per hr.). Period B, intravenous saline injections, total received 420 cc. (3 injections per hr.).

Date.	Time.	Urine.				Plasma sugar.		Hemoglobin.		Temperature.		Remarks.
		Volume.		Glucose.								
		A	B	A	B	A	B	A	B	A	B	
		cc.	cc.	per cent	per cent	per cent	per cent	per cent	per cent	°F.	°F.	
1915												
Dec. 15	1.30 p.m.	31	50	0	0	0.135	0.167	104	113	101.4	101.6	1.50 p.m. started injections.
Period A.	2.10 "	15	9	1.88	0	0.294	0.147		104	101.1	101.3	
Dec. 27	2.30 "	65	15	2.14	0	0.400	0.139	102	98	100.9	101.7	Finished injections.
Period B.	2.50 "	108	38	2.11	0	0.400	0.139	93	81	100.8	101.6	
	3.10 "	27	13	2.31	0	0.358	0.139	94	81	100.8	101.2	
	3.30 "	13	10	1.78	0	0.250	0.162	85	90	101.1	101.3	
	3.45 "	17	14	0.46	0	0.264	0.167	93	80	101.4	101.4	
	4.00 "	26	20	Very faint.	0	0.208	0.152	90	82	101.4	101.2	
	4.15 "	40	21	0	0	0.226	0.183	96		102.2	101.7	

Period A. Total solution injected.................. 420 cc.
" glucose " .... 21 gm.
" urine excreted.................. 311 cc.
" glucose " .... 4.9 gm.
Per cent of dose.................. 23.3 per cent.

Period B. Total solution injected.................. 420 cc.
" urine excreted.................. 140 "

TABLE XXVIII.

Dog C3-86.

Weight 13 kg. Partially depancreatized. Severely diabetic. Intravenous glucose injections, 1 gm. per kg. per hr. in 20 per cent solution (3 injections per hr.).

Date.	Time.	Urine.		Plasma sugar.	Hemoglobin.	Remarks.
		Volume.	Glucose.			
1916		*cc.*	*per cent*	*per cent*	*per cent*	
May 9	2.55 p.m.		0	0.128	99	Started injections.
Period	3.15 "	8	0.57	0.130	95	
A.	3.35 "	3	1.54	0.200	97	
	3.55 "	10	1.08	0.270	90	
	4.15 "	3	3.45	0.227	84	
	4.35 "	3	2.04	0.232	76	Finished injections.
	4.55 "	4	1.76	0.185	74	
	5.30 "	55	0	.		Total solution in-
	5.45 "			0.123	90	jected.......... 130 cc.
	7.00 "	91	0	0.099	99	Total glucose in-
						jected.......... 26 gm.
						Total urine excreted 177 cc.
						Total glucose ex-
						creted.......... 0.44 gm.
						Per cent of dose.... 1.69 per cent.
May 15						10.30 a.m. fed 200 gm. of lard.
Period	3.00 "		0	0.098	90	Started injections.
B.	3.20 "	6	1.79	0.170	90	
	3.40 "	11	0.95	0.170	80	
	4.00 "	11	1.20	0.164	94	
	4.20 "	4	2.15	0.145	100	
	4.40 "	3	2.09	0.137	100	Finished injections.
	5.00 "	5	1.61	0.125	100	
	5.50 "	8	Slight.	0.116	96	Total solution in-
	7.00 "	15	0	0.100	89	jected.......... 130 cc.
	7.30 "	12	0			Total glucose in-
						jected.......... 26 gm.
						Total urine excreted. 75 cc.
						Total glucose ex-
						creted.......... 0.57 gm.
				.		Per cent of dose.... 2.19 per cent.

TABLE XXVIII—*Concluded*.

Date.	Time.	Urine.		Plasma sugar.	Hemoglobin.	Remarks.
		Volume.	Glucose.			
1916		*cc.*	*per cent*	*per cent*	*per cent*	
May 22	11.25 a.m.			0.119	88	Fed 1,200 gm. of lung.
Period	1.50 p.m.	72	0	0.123	88	Started injections.
C.	2.15 "	11	2.38	0.164	88	
	2.35 "	16	2.27	0.185	77	
	2.55 "	9	4.16	0.233	81	
	3.15 "	15	3.85	0.204	80	
	3.35 "	12	3.03	0.200	70	Finished injections.
	3.55 "	12	1.52	0.161	89	
	4.40 "	15	Faint.	0.125	93	4.40 p.m. vomited 400 gm. of undigested lung.
	6.25 "	75	0	0.095	100	Total solution injected.......... 130 cc. Total glucose injected.......... 26 gm. Total urine excreted 165 cc. Total glucose excreted.......... 2.11 gm. Per cent of dose.... 8.10 per cent.

TABLE XXIX.

Dog E5-90.

Weight 10 kg. Period A, very mild diabetes. Glycosuria absent on diet of bread and soup; continuously heavy with addition of 100 gm. of glucose. Period B, moderate diabetes. Glycosuria absent on diet of lung and suet; heavy with addition of 200 gm. of bread. Intravenous glucose injections, 1 gm. per kg. per hr. in 10 per cent solution (4 injections per hr.).

Date.	Time.	Urine.		Plasma sugar.	Remarks.
		Volume.	Glucose.		
1917		*cc.*	*per cent*	*per cent*	
Oct. 25	1.45 p.m.		0	0.164	1.55 p.m. started injections.
Period	2.50 "	5	0.68	0.333	
A.	3.50 "	14	0.57	0.323	4.40 p.m. finished injections.
	4.50 "	95	Faint.	0.213	
	6.15 "			0.119	
					Total solution injected. 300 cc.
					Total glucose injected.. 30 gm.
					Total urine excreted... 114 cc.
					Total glucose excreted. 0.11 gm.
					Per cent of dose....... 0.37 per cent.
Dec. 18	2.45 "		0	0.122	3.00 p.m. started injections.
Period	4.00 "	14	2.25	0.384	
B.	5.00 "	50	4.00	0.500	5.45 p.m. finished injections.
	6.00 "	26	2.60	0.455	
					Total solution injected. 300 cc.
					Total glucose injected.. 30 gm.
					Total urine excreted... 90 cc.
					Total glucose excreted. 3.0 gm.
					Per cent of dose....... 10 per cent.

TABLE XXX.

Dog D4-52.

Weight, Period A, 10.75 kg.; Period B, 11.7 kg.; Period C, 12 kg. Glycosuria absent on bread and soup diet to Oct. 10, 1917. Absent thereafter on 500 gm. of lung and 100 gm. of suet, with hyperglycemia. Intravenous glucose injections, 1 gm. per kg. per hr. in 10 per cent solution (4 injections per hr.), based on normal weight of 12 kg.

Date.	Time.	Volume.	Urine. Glucose.	Plasma sugar.	Remarks.
1917		*cc.*	*per cent*	*per cent*	
Aug. 6 Period A.	Before injection.		0	0.218	
	End 1st hr.	16	2.64	0.500	
	" 2nd "	16	5.08	0.715	
	" 3rd "	54	2.84	0.475	Plasma sugar imme-
	" 4th "	52	1.54	0.590	diately after injec-
	" 5th "	76	0.74	0.415	tion.............. 0.770 per cent.
	" 6th "	78	1.81	0.270	Total solution in-
	" 7th "	94	1.45	0.202	jected............ 828 cc.
	" 8th "	82	0.82	0.125	Total glucose in-
	1 hr. after injection.	53	0.19	0.128	jected............ 96 gm. Total urine excreted.. 563 cc.
	2 hrs. after injection.	12	Faint.	0.130	Total glucose ex- creted............ 7.8 gm.
	3 hrs. after injection.	29	0	0.133	Per cent of dose...... 8.1 per cent.
Nov. 19 Period B.	Before injection.		0	0.169	
	End 1st hr.	36	1.93	0.555	
	" 2nd "	68	3.08	0.555	
	" 3rd "	82	1.49	0.435	
	" 4th "	132	0.79	0.370	
	" 5th "	98	0.39	0.370	Total solution in-
	" 6th "	126	0.28	0.356	jected............ 1,200 cc.
	" 7th "	127	0.55	0.370	Total glucose in-
	" 8th "	105	0.39	0.370	jected............ 120 gm.
	" 9th "	105	0.36	0.384	Total urine excreted.. 1,035 cc.
	" 10th "	101	· 0.42	0.322	
	1 hr. after injection.	45	Faint.	0.156	Total glucose ex- creted............ 7.7 gm.
	2 hrs. after injection.	10	0	0.147	Per cent of dose..... 6.4 per cent.

TABLE XXX—*Concluded.*

Date.	Time.	Urine. Volume.	Urine. Glucose	Plasma sugar.	Remarks.
1918		*cc.*	*per cent*	*per cent*	
Feb. 19 Period C.	Before injection.		0	0.145	
	End 1st hr.	20	4.77	0.417	
	" 2nd "	55	5.13	0.476	
	" 3rd "	114	3.39	0.500	
	" 4th "	109	3.45	0.525	Total solution injected............ 960 cc.
	" 5th "	102	3.40	0.500	
	" 6th "	140	2.78	0.475	Total glucose injected........... 96 gm.
	" 7th "	90	4.35	0.455	
	" 8th "	87	3.23	0.384	Total urine excreted.. 778 cc.
	1 hr. after injection.	43	0.74	0.294	Total glucose excreted........... 25.7 gm.
	2 hrs. after injection.	19	Very faint.	0.170	Per cent of dose..... 26.8 per cent.

TABLE XXXI.

Dog D4-69.

Glycosuria absent on bread and soup to Oct. 5, 1917; thereafter absent on 500 gm. of lung and 100 gm. of suet, with development of hyperglycemia. Intravenous glucose injections, 1.3 gm. per kg. per hr. in 10 per cent solution (4 injections per hr.), based on 15 kg. weight.

Time.	Period A. Aug. 6, 1917. Weight 16.2 kg.			Period B. Dec. 4, 1917. Weight 14.2 kg.			Period C. Feb. 19, 1918. Weight 15.5 kg.		
	Urine.		Plasma sugar.	Urine.		Plasma sugar.	Urine.		Plasma sugar.
	Volume.	Glucose.		Volume.	Glucose.		Volume.	Glucose.	
	cc.	*per cent*	*per cent*	*cc.*	*per cent*	*per cent*	*cc.*	*per cent*	*per cent*
Before injection		0	0.106		0	0.104		0	0.154
End 1st hr.	20	1.56	0.278	100	2.84	0.715	46	4.83	0.625
" 2nd "	30	2.87	0.294	144	4.45	0.990	214	4.00	0.800
" 3rd "	64	1.71	0.312	222	3.65	0.990	252	4.35	1.000
" 4th "	106	0.76	0.270	300	2.80	1.000	274	3.70	1.180
" 5th "	110	0.42	0.218	220	2.93	0.844	281	4.25	1.110
" 6th "	76	0.53	0.204	265	2.43	0.844	270	4.55	1.050
" 7th "	128	0.51	0.183	222	2.71	0.625	222	4.55	1.000
" 8th "				216	3.82	0.715	278	5.13	0.950
1 hr. after injection	90	Faint.	0.082	60	3.60	0.370	78	5.88	0.525
2 hrs. "	82	0	0.098	66	0.49	0.217	10	5.13	0.322

Period A. Total solution injected 1,560 cc.   Period B. Total solution injected 1,560 cc.   Period C. Total solution injected 1,560 cc.
  " glucose " 156 gm.   " glucose " 156 gm.   " glucose " 156 gm.
  " urine excreted... 706 cc.   " urine excreted... 805 cc.   urine excreted...1,925 cc.
  " glucose " 4.5 gm.   " glucose " 55.2 gm.   " glucose " 80.4 gm.
  Per cent of dose....... 2.87 per cent.   Per cent of dose....... 35.4 per cent.   Per cent of dose....... 51.5 per cent.

TABLE XXXII.

Percentage of Total Intravenously Injected Dose of Glucose Excreted by Normal Dogs.

Dosage per kg. per hr.	1 gm. in 10 per cent solution (3 injections per hr.).	1 gm. in 20 per cent solution (3 injections per hr.).			1 gm. in 5 per cent solution (2 injections per hr.).	1.5 gm. in 20 per cent solution (3 injections per hr.).								1.5 gm. in 10 per cent solution (4 injections per hr.).	1.5 gm. in 7.5 per cent solution (2 injections per hr.).	2 gm. in 5 per cent solution (continuously).
Duration	For 2 hrs.	For 3 hrs.	For 10 hrs.	For 3 hrs.	For 2 hrs.	For 2 hrs.	For 2 hrs.	For 2 hrs.	For 3 hrs.	For 3 hrs.	For 3 hrs.	For 3 hrs.	For 4 hrs.	For 9 hrs.	For 2 hrs.	For 4 hrs.
Table No.	I	II	III	VIII	IV (A)	V (A)	VI	V (B)	VII	IX	X	XI	XII	XIII	IV (B)	XIV
Per cent of total dose excreted	0.73	2.16	1.48	8.40	0	2.28	4.40	1.41	1.60	1.50	1.50	1.28	1.40	1.88	2.27	5.90
Per cent of total dose excreted for first 2 hrs.			0.85											Trace.		
Per cent of total dose excreted for first 3 hrs.			1.40											0.96		

TABLE XXXIII.

Percentage of Total Dose of Intravenously Injected Glucose Excreted by Partially Depancreatized Dogs.

Table No.	No diabetes		Very mild diabetes			Mild diabetes			Moderate diabetes			Severe diabetes		
	XV	XVI	XVII (A)	XVIII	XIX (A)	XX	XXI	XXII	XXIII	XXIV	XXV	XXVI	XXVII	XXVIII (A)
Dosage per kg. per hr.	1 gm. in 20 per cent solution (3 injections per hr.).	1.5 gm. in 5 per cent solution (3 injections per hr.).	1.2 gm. in 5 per cent solution (continuous).	1.5 gm. in 5 per cent solution (3 injections per hr.).	1 gm. in 20 per cent solution (3 injections per hr.).	1 gm. in 5 per cent solution (3 injections per hr.).	1 gm. in 20 per cent solution (3 injections per hr.).	1 gm. in 20 per cent solution (3 injections per hr.).	1 gm. in 20 per cent solution (4 injections per hr.).	1 gm. in 20 per cent solution (3 injections per hr.).	1 gm. in 10 per cent solution (4 injections per hr.).	1 gm. in 5 per cent solution (4 injections per hr.).	1.5 gm. in 5 per cent solution (3 injections per hr.).	1 gm. in 20 per cent solution (3 injections per hr.).
Duration.	For 3 hrs.	For 2 hrs.	For 3 hrs.	For 1 hr.	For 2 hrs.	For 3½ hrs.	For 2 hrs.	For 3 hrs.	For 2 hrs.	For 2½ hrs.	For 2 hrs.	For 2½ hrs.	For 1 hr.	For 2 hrs.
Per cent of total dose excreted.	2.2	5.6	14.1	10.5	3.02	0	8.2	26.4	20.4	19.8	22.2	20.5	23.3	1.69

A NEW ELECTRODE FOR USE IN CLINICAL ELECTRO-CARDIOGRAPHY.*

By ALFRED E. COHN, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research.)

It has become sufficiently clear that if in taking human electrocardiograms there is overshooting of the string, a high resistance has probably developed at the site at which the electrodes have been applied to an individual. Overshooting is, of course, undesirable because it deforms the curve. A high metallic resistance interposed in the circuit does not cause this defect. The deformity is seen either when an added resistance is introduced into the circuit or when a current passes. In the latter instance the overshooting is the greater,

TABLE 1.

Resistances Obtained with Various Electrodes.

Order of Taking Electrocardiograms	Type of Electrode	Figure	Resistance Before Salt Rub			Order of Taking Electrocardiograms	Figure	Resistance After Salt Rub		
			Lead 1 Ohms	Lead 2 Ohms	Lead 3 Ohms			Lead 1 Ohms	Lead 2 Ohms	Lead 3 Ohms
1	Plate	..	2,300	4,500	4,000	6	6	2,300	2,300	1,400
2	Lead	3	2,000	2,300	2,300	5	4	1,000	1,000	1,000
3	Immersion	..	4,000	3,000	3,400	4	5	2,500	2,500	2,000
7	German silver	..	.....	.....	.....	..	7	1,400	1,400	1,400

the greater the voltage. It has likewise become sufficiently clear, that if the resistance in the string-patient circuit is reduced below 2,000 ohms, the overshooting does not take place. It makes no difference so far as electrocardiography is concerned what is the reason of the overshooting—whether due to polarization at the electrodes or to a capacity effect in the cutaneous tissues as Pardee[1] has suggested.

* The occasion for making this study was the construction of the electrodes described in the text. The credit for devising them is due to Robert Neübuck, technician in this department. The satisfaction which their use has given has prompted the controlling tests which are now reported.

1. Pardee, H. E. B.: An error in the electrocardiogram arising in the application of the electrode, Arch. Int. Med. 20: 161 (Aug.), 1917.

Provided, then, that the resistance is low, the form of electrode used is probably a matter of indifference.   The electrode of choice is the one easiest to apply, easiest to keep in order, easiest and cheapest to make.   At the Hospital of The Rockefeller Institute the electrode in use is made of a strip of lead foil[2] 7.5 cm. wide by 22.0 cm. long, and
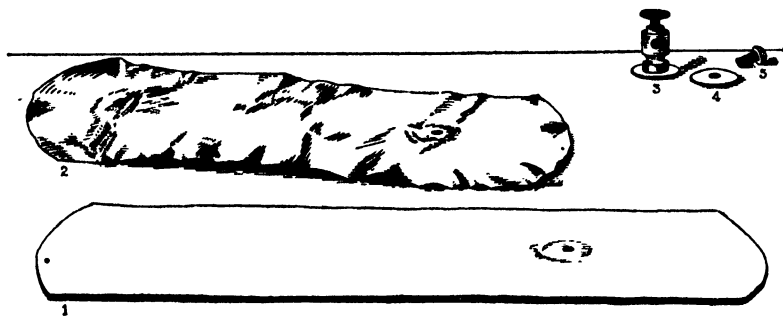
FIG. 1.—Electrode in use at Hospital of Rockefeller Institute.   The lead foil, rubber sheet and binding post are shown separately.

FIG. 2.—The electrode as assembled and ready for use.

of a strip of rubber sheet 9.0 cm. wide by 30.0 cm. long.   The two strips are fastened together about 8.0 cm. from one end by a brass screw, brass binding post and washers, in the manner shown in Figures 1 and 2.   No soldering is necessary.   The manner of fastening permits the repeated use of the remaining portion of the lead foil if

2. The lead foil is an alloy of lead and tin known as roentgen-ray protection foil.   The gage used is B. & S. 31.   It is supplied in rolls 12 inches wide.   The rubber strip is cut from rubber matting 1/16 inch thick, 3 feet wide, which may be obtained from the New York Belting and Packing Company.

a break takes place at the binding post. This accident after prolonged use is of course unavoidable.

In order to test and to establish the usefulness of these electrodes, electrocardiograms made with them (Figs. 3 and 4) were compared with others taken with immersion-non-polarizable electrodes (Fig. 5), with the so-called plate electrodes (Fig. 6), and with German silver
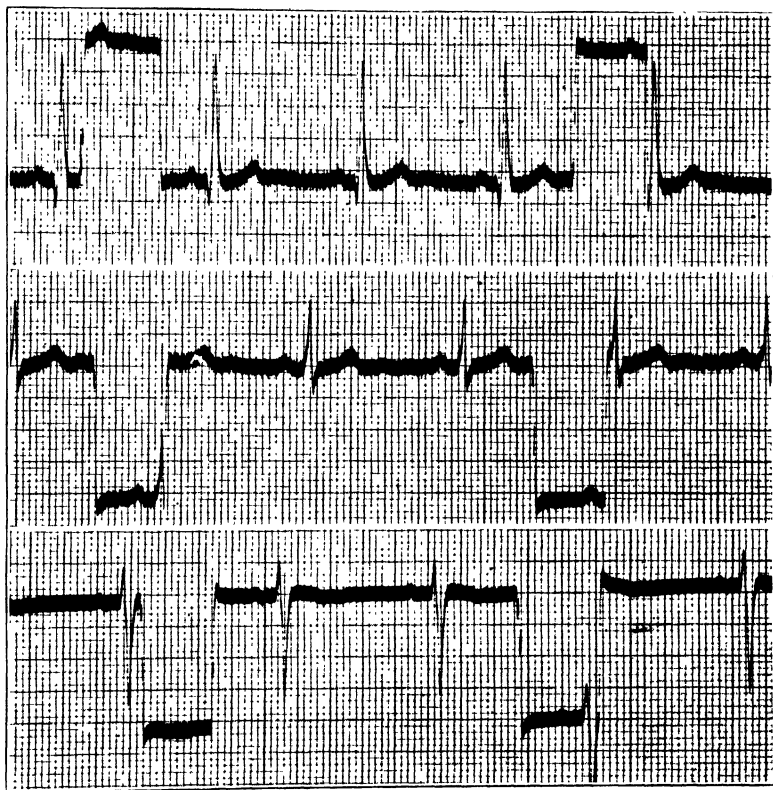
FIG. 3.--In all the electrocardiograms divisions of the ordinates equal 0.1 millivolt; divisions of the abscissae equal 0.04 second. The three usual leads are arranged from above downward. In each figure there are two deflections of about 20.00 mm. and of about 0.4 to 0.5 second duration. These are deflections caused by throwing 20 ohms into the circuit. This electrocardiogram was taken with lead electrodes before rubbing the skin with salt solution. The resistance of Lead 1 is 2,000; of Lead 2, 2,300; of Lead 3, 3,300 ohms.

TABLE

Measurements of the

Electrodes	Figure		P₁*	P₂	P₃	Q₁	Q₂	Q₃
			Mm.	Mm.	Mm.	Mm.	Mm.	Mm.
Lead......	3	Range	1.0–1.1	1.0	0.2 –0.4	2.75–3.1	—	—
Lead......	4		0.9–0.1	1.05–1.15	0.50–0.62	2.4 –2.9	—	—
Immersion.	5		1.00	1.0	0.43–0.6	2.4 –2.65	—	↙ 4
Plate......	6		1.0–1.5	1.2–1.5	0.6 –0.8	2.5 –3.0	—	—
German silver....	7		0.75–1 2	0.9–1.0	0.8 –0.1	2.55–2.8	—	—
Lead......	3	Observed average	1.05	1.0	0.33	2.85	—	—
Lead......	4		0 95	1 09	0.57	2.68	—	—
Immersion.	5		1.0	1.0	0.50	2.53	—	—
Plate......	6		1.07	1.31	0.71	2.72	—	—
German silver....	7		0.91	0.97	0.92	2.69	—	—
			Mm. %	Mm. %	Mm. %			
Lead......	3	Observed standard deflection†	21.51 = 107.5	21 5 = 107.5	20.13 = 100.65	Same as P₁	Same as P₃	Same as P₂
Lead.....	4		20 3 = 101.5	21.01 = 105 05	21.3 = 106.5			
Immersion.	5		20 53 = 102.65	20.59 = 102.95	20.57 = 102.85			
Plate......	6		21.08 = 105.4	21.05 = 105.25	20.43 = 102.15			
German silver....	7		20.16 = 100.8	21.08 = 105.4	21.28 = 106.4			
Lead......	3	Corrected average	0 98	0.93	0.33	2.65	—	—
Lead......	4		0.94	1.04	0.54	2.64	—	—
Immersion.	5		0.97	0.97	0.49	2.47	—	—
Plate......	6		1.02	1.25	0.70	2.58	—	—
German silver....	7		0.90	0.93	0.87	2.67	—	—

* P₁ = P wave in Lead 1; P₂ = P wave in Lead 2, etc.

† The standard deflection when 20 ohms are thrown into string circuit is 20.0 mm. If 20.0 expressed in per cent. This figure is used for correcting the observed average to 100 per

2.

Waves in Figures 3 to 7.

R₁	R₂	R₃	S₁	S₂	S₃	T₁	T₂	T₃
Mm.	Mm.	Mm.	Mm.	Mm.	Mm.	Mm.	Mm.	Mm.
18.4-19.0	9.6 -10.1	4.25-4.7	--	2.7-3.0	15 1 -15 9	1 95-2.1	1.9 -2.36	-0.65--0.7
17.4-18.26	8.7 -9.13	4.25-4 5	—	1.5-2 0	13.8 -15.0	2.0 -2.3	1.85-2.15	-0.5 --0.8
15.8-17.0	8 6 -8.95	4.1 -4 85	--	2 2 -2 65	12.7 -14.65	2.1 -2.2	1.75-2.1	-0.4 --0.67
18.2-19.75	8.42-8.85	3 85 4.25	—	1 2 -2 0	13 75-15.4	2.5 -2.72	1.85-2.35	-0.6 --0.87
17.7-19.2	8 55 8.9	3.95-4 2	--	1.0 -1.17	13 82-15.27	2.25-2.72	2.1 -2.35	-0.35--0.45
18.7	9.90	4 46	—	2.87	15 55	2.02	2.08	- 0.68
17.68	8 97	4 44	—	1 67	14.64	2.18	2.00	- 0.60
16.45	8.77	4.44	—	2.43	13.73	2.12	1.98	- 0.50
19.04	8.70	4.03	—	1 61	14.88	2.56	2.03	- 0.72
18.75	8.76	4.11	—	1.06	14.66	2.51	2.22	- 0.38
Same as P₁	Same as P₂	Same as P₃	Same as P₁	Same as P₂	Same as P₃	Same as P₁	Same as P₃	Same as P₂
17.39	9.21	4.43	—	2.67	15.45	1.88	1.94	- 0.68
17.43	8.55	4.17	—	1.59	13.75	2.15	1.90	- 0.56
16.03	8.52	4.32	—	2.37	13.36	2.07	1.93	- 0.49
18.06	8.27	3.95	—	1.53	14.57	2.43	1.94	- 0.71
18.59	8.32	3.87	—	1.01	13.78	2.49	2.11	- 0.36

mm. equals 100 per cent., a deviation from this height of the observed deflection may be cent.

electrodes (Fig. 7). The plate electrodes were fashioned after the manner of those introduced by the Cambridge Instrument Company; those of German silver after the pattern described by Williams;[3] the nonpolarizable ones in the manner which is usual when the extremities
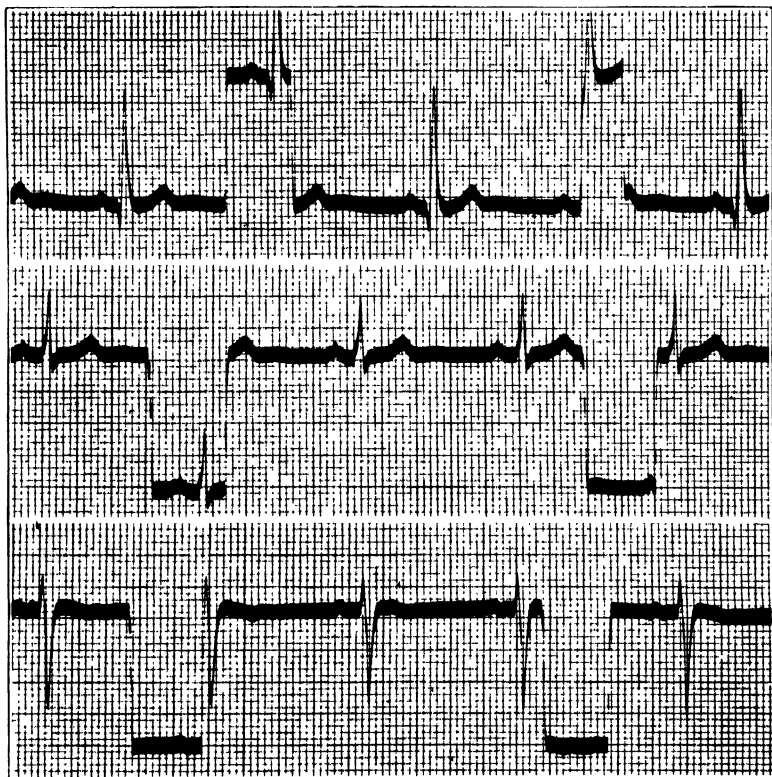
FIG. 4.—The same after rubbing the skin with salt solution. The resistance of Lead 1 is 1,000; of Lead 2, 1,000; and of Lead 3, 3,100 ohms.

are immersed. The electrocardiograms were taken of a single individual at a single sitting in the order shown in Table 1.

After exposures noted in the first column were made (Table 1), the arms and leg of the patient were rubbed gently with warm satu-

3. James, W. B., and Williams, H. B.: The electrocardiogram in clinical medicine, Am. J. M. Sc. 140: 408, 1910.

rated salt solution; then those recorded in the second column were taken. Taking all the electrocardiograms occupied about one hour. Slight overshooting is seen in Figure 3 (the lead electrode before
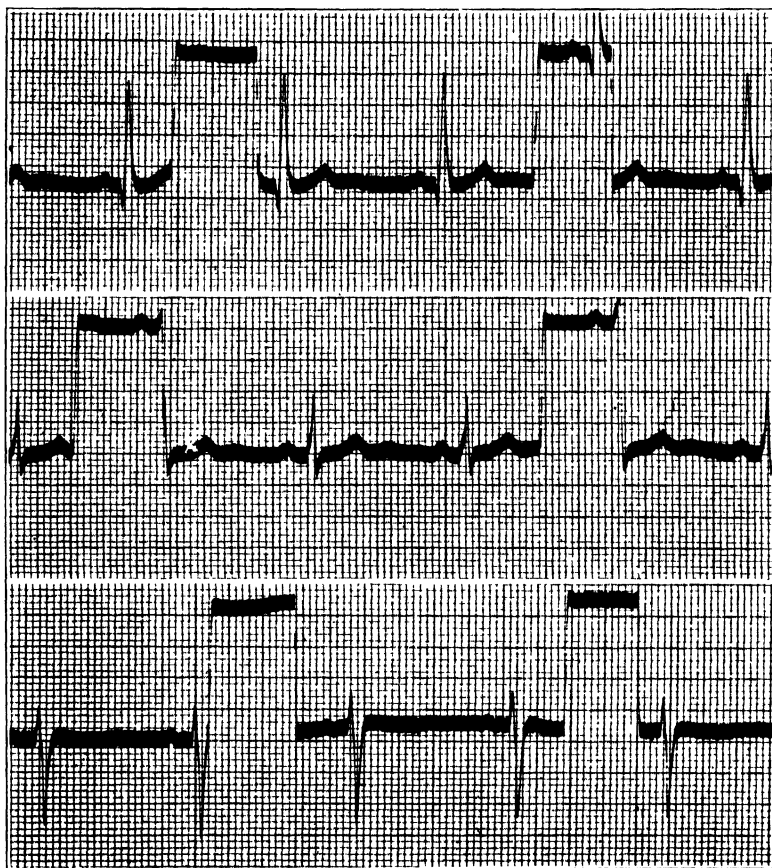
FIG. 5.—This electrocardiogram was taken with immersion nonpolarizable electrodes after rubbing the skin with salt solution. The resistance of Lead 1 is 2,500; Lead 2, 2,500; and Lead 3, 2,500 ohms.

rubbing), and in Figure 5 (the immersion nonpolarizable electrode). On the whole, the resistance was highest when these two were taken. In comparing them, however, it is not the object to show the relation

of resistance to the degree of overshooting, but to show the fact that the electrocardiograms, irrespective of the form of the electrodes, are closely comparable at resistances of this magnitude.[4]

That the curves are comparable is shown (Table 2) by the fact that the height of a wave in one lead, the R wave for instance in Lead 1, differs little from the other R waves in the same lead, that is to say, the range of variation is small (Table 3). It is also shown (Table 2) that if a wave is small when taken with one electrode (for instance, P_1[5] taken with the German silver electrode), it does not follow that

TABLE 3.

Limits of Ranges of the Corrected Averages.

Wave	Lead	Limits	Range
P	1	0.90 and 1.02 mm.	0.12 mm.
P	2	0.93 to 1.25	0.32
P	3	0.33 to 0.87	0.54
Q	1	2.47 to 2.67	0.20
R	1	16.03 to 18.59	2.56
R	2	8.27 to 9.21	0.94
R	3	3.87 to 4.43	0.56
S	2	1.01 to 2.67	1.66
S	3	13.36 to 15.45	2.09
T	1	1.88 to 2.49	0.61
T	2	1.90 to 2.11	0.21
T	2	0.36 to 0.71	0.35

another wave (e. g. R_1) taken with the same electrode is also small, when compared with like waves (R_1) taken with other electrodes. (R_1 taken with the German silver electrode for instance is the tallest of the R waves in Lead 1.) That in the same lead, the height of a given wave (R_1 for instance) varies is due probably to the influence of respiration.

In view of the practical identity of the heights of the waves all the electrodes described may be used interchangeably. The electrodes

4. A comparison of curves taken at higher resistances is not essential in this study, for it is admitted that overshooting occurs at higher resistances, and likewise that the amount of overshooting differs with different metals. The lead electrodes are perhaps more satisfactory in this respect than other similar ones.

used by us are recommended because their construction is at least as simple as any other, and of the electrodes which are fastened to the limb they are smaller and easier to apply. They are by far the most comfortable, and can therefore be kept in place during pro-
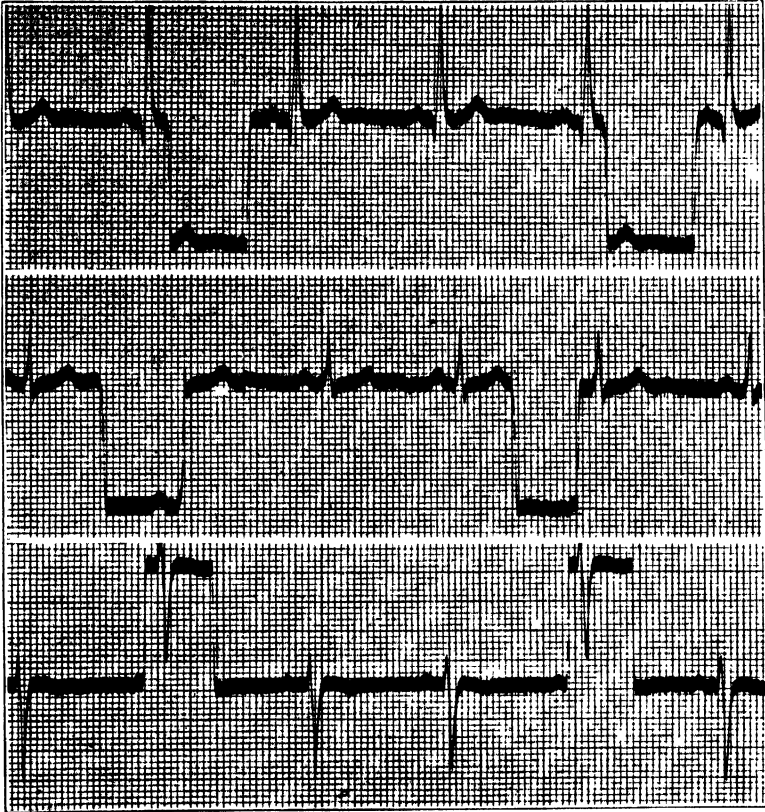
FIG. 6.—This electrocardiogram was taken with plate electrodes after rubbing the skin with salt solution. The resistance of Lead 1 is 2,300; of Lead 2, 2,300; of Lead 3, 1,400 ohms.

longed observations. They answer the requirement that with them a low resistance can be attained, and a low resistance is the most important criterion in establishing the usefulness of electrodes. It is by no means uncommon to obtain resistances as low as 500 ohms.
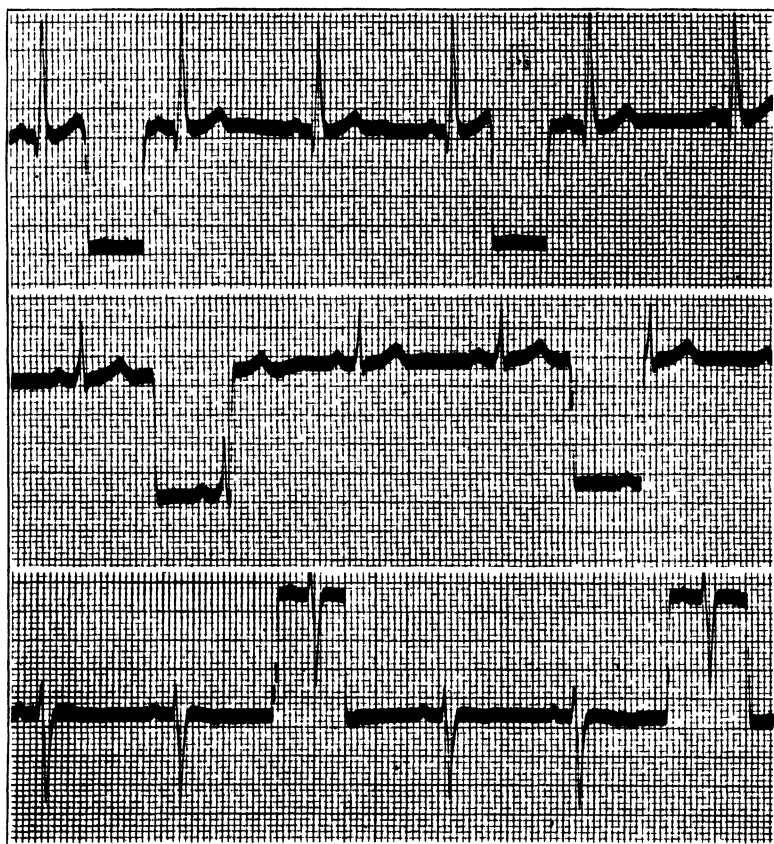
FIG. 7.—This electrocardiogram was taken with German silver electrodes after rubbing the skin with salt solution. The resistance of Lead 1 is 1,400; of Lead 2, 1,400; of Lead 3, 1,400 ohms.

SUMMARY.

With all electrodes, electrocardiograms are deformed by over-shooting of the string, when the resistance developed at the site of the application is high.

Electrodes of simple construction are described. Electrocardiograms taken with them are identical with those taken by other electrodes, when the resistances are below 2,000 and comparable.

EXPERIMENTS ON CARBOHYDRATE METABOLISM AND DIABETES.

II. THE RENAL THRESHOLD FOR SUGAR AND SOME FACTORS MODIFYING IT.

BY FREDERICK M. ALLEN AND MARY B. WISHART.

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Attention was called in the preceding communication[1] to the importance of renal permeability in determining the apparent tolerance for sugar. The present group of papers, together with that of Palmer[2] on tissue permeability, are fragments of a research originally planned on the passage of sugar through different body membranes in diabetic and non-diabetic conditions. Publication of the following incomplete observations is ventured because of the apparent suggestiveness of some of them.

No detailed survey of the literature on the permeability of the kidney for sugar will be attempted. Some of the older literature was reviewed by Allen,[3] and the principal facts bearing on the present topic may be summarized as follows: (*a*) a brief glycosuria is attended by a lowering of the sugar threshold of the normal kidney, so that as the hyperglycemia is subsiding sugar continues to pass into the urine with a lower level of blood sugar than that which is necessary to cause glycosuria at the outset; (*b*) prolonged glycosuria or hyperglycemia is attended with an elevation of the sugar threshold, so that glycosuria may remain absent with a blood sugar level considerably higher than that at which sugar excretion ordinarily occurs; (*c*) renal abnormalities, either spontaneous diseases or drugs and other

[1] Allen, F. M., and Wishart, M. B., *J. Biol. Chem.*, 1920, xlii, 415.

[2] Palmer, W. W., *J. Biol. Chem.*, 1917, xxx, 79.

[3] Allen, F. M., Studies concerning glycosuria and diabetes, Cambridge, 1913, 44, 384 ff., 541.

agencies, may either increase or diminish the permeability.   Epstein and Baehr[4] and Woodyatt[5] have emphasized the importance of blood volume in addition to the percentage concentration of sugar for governing the excretion.   This, however, is presumably only one of many factors influencing renal permeability.   The present observations were accompanied with hemoglobin and corpuscle percentage determinations, as illustrated in the following and other papers, and were not explainable by blood volume changes as far as could be judged by these methods.

Benedict and Osterberg[6] have recently placed this subject on a new basis, by devising a quantitative method for the sugar in normal urine.   With this procedure there is no renal threshold for glucose, and various metabolic conditions can be more accurately studied in their influence upon the kind as well as the quantity of urinary carbohydrate.   Two excuses may be offered for presenting observations based upon the older idea of the renal threshold and a fixed distinction between glycosuria and its absence as opposed to the newer concept of normal and pathological variations of glycuresis.   One is that so many matters of experimental and clinical importance have been connected with the excretion of sugar according to the older standard, that though this may not be subject to modification it can scarcely lose its significance altogether.   The other is the likelihood that all valid standards will be found to agree, and that the finer observations with the new method may in general confirm the cruder ones with the old.   The present findings may therefore at least suggest causes of altered renal permeability which are worth investigating by the improved method.

A large number of parallel analyses of urine by copper reduction according to Benedict and of blood by the Benedict picric acid method have been performed in the course of the entire investigation, but relatively few are suitable for fixing the threshold or permeability. Consideration is therefore limited chiefly to forty-nine dogs, from

[4] Epstein, A. A., and Baehr, G., *J. Biol. Chem.*, 1914, xviii, 21.   Epstein, A. A., *Proc. Soc. Exp. Biol. and Med.*, 1915–16, xiii, 67; *Am. J. Med. Sc.*, 1917, cliv, 103.

[5] Woodyatt, R. T., *The Harvey Lectures*, 1915–16, xi, 326.

[6] Benedict, S. R., and Osterberg, E., *J. Biol. Chem.*, 1918, xxxiv, 195.

which parallel samples of blood and urine were obtained at 15 minute or other suitable intervals, or the urine alone was collected frequently and a blood sample taken as soon as a reducing reaction appeared or disappeared. The experiments were seldom performed directly for this purpose, but by bearing this point in mind it was easily included as an incidental observation in experiments of various kinds. The number of observations is multiplied by the fact that they were repeated in all the above dogs under different experimental conditions. The animals most often mentioned are those in which the changes were followed through successive stages of diabetes. The results are briefly summarized under the respective types of experiments (fasting, different injections, feedings, etc.), and the individual dogs under each heading are arranged in order according to the gradations of tolerance or diabetes.

The degree of diabetes is naturally something which cannot be defined exactly, but for this purpose dogs were considered severely diabetic if they showed glycosuria on protein diet, and mildly diabetic if considerable quantities of carbohydrate were necessary for glycosuria. Such diabetes was merely "potential" except on test days, because active symptoms were kept absent as a rule by appropriate diets. Other dogs had undergone removal of $\frac{3}{4}$ or more of the pancreas, but not enough to produce diabetes. These and the normal dogs were on bread and soup diets. The others were on such protein diets as were necessary for sugar freedom, with addition of fat sometimes as specified.

Fasting Plasma Sugars.

The dogs were fed between 10 a.m. and noon, and the blood samples designated as "fasting" were taken before feeding the next day. Table I gives a summary of the results.

As the conditions specified preclude any glycosuria, this group naturally includes no observations of threshold. The plasma sugar values found for the normal animals agree with those in the literature and with numerous others in the course of this research. Those of the partially depancreatized non-diabetic animals are entirely similar, there being no tendency to fasting hyperglycemia from the removal of pancreas tissue to any point short of diabetes. They may,

however, be more readily subject to hyperglycemia from slight excitement or other disturbance, thus accounting for the maximum of 0.154 per cent. The values are observed to rise with increasing severity of diabetes. The important point in the present connection is that these levels were reached and maintained for long periods without glycosuria. As heavy glycosuria always accompanies such values normally, it must be concluded that there is a marked elevation of the sugar threshold with increasing severity of diabetes. This corresponds to the observations in the great majority of human cases.

The rôle of the long continued excess of sugar itself will be discussed below. Some part may be attributed to it in most of the above animals, as also in most human cases, because of the history of long

TABLE I.

Condition.	No. of dogs.	No. of analyses.	Plasma sugar.		
			Maximum.	Minimum.	Average.
			per cent	*per cent*	*per cent*
Normal......................................	10	14	0.121	0.070	0.108
Partially depancreatized non-diabetic............	10	46	0.154	0.065	0.109
Mild diabetes.................................	11	43	0.200	0.084	0.143
Severe diabetes...............................	18	51	0.357	0.109	0.196

continued glycosuria or hyperglycemia in most of them. That a very prolonged excess of sugar, however, is not essential to such elevation of the threshold is proved by the records of certain animals.

Dog D4-28 was previously described.[7] $\frac{11}{12}$ of the pancreas was removed[8] on Sept. 28, 1916, and the dog was radically undernourished, so that hyperglycemia was brief and rare. Nevertheless in a feeding test on Dec. 1, 1917, the plasma sugar went to 0.216 per cent without glycosuria. It may be worth noticing that the diet had contained considerable fat.

Dog F6-08, a male Dalmatian aged 3 years, weighing 15 kilos, on Dec. 6, 1917, underwent removal of 27.9 gm. of pancreatic tissue, leaving a remnant estimated at 1.4 gm. ($\frac{1}{20}$). With fasting there was no glycosuria, but the plasma sugar on the morning of Dec. 8 was 0.179 per cent. Additional bits of pancreatic tissue were removed for microscopic examination on Dec. 14 and 19. 100 gm. of bacon grease were fed daily after Dec. 24, no other food being given. Dec. 31, at a weight of 8.5 kilos, the plasma sugar was 0.256 per cent without glycosuria.

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[7] Allen, F. M., *J. Exp. Med.*, 1920, xxxi, 581–583.
[8] All operations were performed under ether anesthesia.

Dog F6-09, a shaggy male mongrel aged 8 years, weighing 18.75 kilos, on Dec. 6, 1917, underwent removal of 36.2 gm. of pancreatic tissue, leaving a remnant estimated at 2.6 gm. ($\frac{1}{14}$). With fasting there was no glycosuria, and the plasma sugar was 0.242 per cent on Dec. 8 and 0.192 per cent on Dec. 10. Without food and without glycosuria, the plasma sugar had risen by Dec. 13 to 0.313 per cent.

Dog F6-11, a female mongrel aged 6 years, weighing 19 kilos, on Dec. 19, 1917, underwent removal of pancreatic tissue weighing 26.3 gm., leaving a remnant estimated at 1.9 gm. ($\frac{1}{14}$ to $\frac{1}{15}$). With fasting and continuous absence of glycosuria, the plasma sugar on Dec. 26 was 0.278 per cent and on Dec. 27, 0.357 per cent.

Subcutaneous Glucose Injections.

Glucose was injected subcutaneously in fasting dogs in 30 per cent solution. Satisfactory threshold figures were obtained in only three partially depancreatized non-diabetic animals and one with mild diabetes.

Dog B2-00, weighing 14 kilos (at a stage when removal of about 1 gm. of additional pancreatic tissue was found necessary to produce diabetes), had a fasting plasma sugar of 0.065 per cent, and received 3 gm. of glucose per kilo subcutaneously. Slight glycosuria occurred with 0.163 per cent of plasma sugar, and a trace could still be detected when the plasma sugar had fallen to 0.118 per cent.

Dog B2-01, with a similar weight and condition, had a fasting plasma sugar of 0.099 per cent, and received 4 gm. of glucose per kilo subcutaneously. With the first trace of glycosuria the plasma sugar was found to be 0.151 per cent. 5 months later the same dose was given. Starting with a fasting plasma sugar of 0.106 per cent, with the first trace of glycosuria the plasma sugar was found to be 0.147 per cent. The threshold had thus apparently not changed with time in a non-diabetic dog on bread diet.

Dog B2-61, weighing 5 kilos and possessing $\frac{1}{4}$ of the pancreas, received 3 gm. of glucose per kilo and showed almost a titratable glycosuria when the plasma sugar reached 0.147 per cent (from a fasting level of 0.106 per cent).

Dog B2-79, weighing 15 kilos, in the stage of mild diabetes, with a remnant of $\frac{1}{4}$ of the pancreas, was kept nearly constantly free from glycosuria on a diet of beef lung, and 5 months after operation received 5 gm. of glucose per kilo subcutaneously. Starting with fasting plasma sugar of 0.105 per cent, a strong trace of sugar appeared in the urine with a plasma sugar of 0.182 per cent. The threshold thus seemed slightly elevated as compared with the non-diabetic animals.

Intravenous Glucose Injections.

With the method of discontinuous injections described in the preceding paper,[1] the threshold of disappearance of glycosuria after the injections are finished is all that can be determined accurately.

Three normal dogs were thus observed, which received 1.5 gm. of glucose per kilo per hour. In one of these glycosuria disappeared while the plasma sugar fell from 0.173 to 0.150 per cent, and in another while the plasma sugar fell from 0.154 to 0.143 per cent. In the third there was a very faint urinary reaction at 0.167 per cent of plasma sugar, and with completely negative urine the plasma sugar was down to 0.128 per cent.

Dog B2-00, partially depancreatized just short of diabetes, received similar dosage and became aglycosuric as the plasma sugar fell from 0.145 to 0.130 per cent.

Dog B2-01, with very mild diabetes, showed a very low threshold as noted in the preceding paper,[9] the urine giving a slight copper reduction during this time while the successive plasma sugars were 0.084, 0.087, and 0.116 per cent.

Three other dogs with slightly greater diabetic tendency showed thresholds between 0.140 and 0.123 per cent, between 0.228 and 0.200 per cent, and between 0.212 and 0.200 per cent, respectively. Here the first dog with the lowest threshold had the smallest pancreas remnant but had also been kept freest from glycosuria and hyperglycemia.

The concentration of the solutions used in the above mentioned tests ranged from 5 to 20 per cent, but showed no constant relation to the different thresholds of disappearance of glycosuria. On the other hand, comparisons may be made with identical doses at different stages of diabetic progress in a dog previously described.[10] Here the thresholds are not exact, because the hour intervals are too long for accuracy, but the comparative relation holds. The table in the reference cited shows that on August 6, 1917 (3 days after the final pancreas operation which produced very mild diabetes), during 2 hours following the last injection there were traces of glycosuria with plasma sugars of 0.125, 0.128, and 0.130 per cent, successively.

[9] Allen and Wishart,[1] p. 439.
[10] Allen, F. M., *J. Exp. Med.*, 1920, xxxi, 567.

Glycosuria was then absent in the next hour during which the plasma sugar rose to 0.133 per cent (indicating disappearance of the special glycosuric tendency which persists for a time during the decline of a glycosuria). On November 19, 1917, there was only a faint trace of glycosuria in the 1st hour after injections with plasma sugar falling from 0.322 to 0.156 per cent, and none in the next hour with plasma sugar between 0.156 and 0.147 per cent. On February 19, 1918, glycosuria disappeared during the 2nd hour after injections with the plasma sugar somewhere between 0.294 and 0.170 per cent, for there was a bare trace of copper reaction in the urine of this hour and none immediately thereafter. Another comparison also can be made between these three dates, by consideration of the fact that the blood analyses during the injection periods were always performed 15 minutes after an injection, just before the next injection. They are thus minimum values, and though not establishing a threshold afford one basis of comparison. The table mentioned shows a change in sugar excretion on this basis. For example, at the 6th and 7th hours on August 6, with plasma sugars of 0.270 and 0.202 per cent, the glycosuria was 1.81 and 1.45 per cent. At the 6th and 7th hours on November 19, with plasma sugars of 0.356 and 0.370 per cent, the glycosuria was 0.28 and 0.55 per cent. At the 6th and 7th hours on February 19, with plasma sugars of 0.475 and 0.455 per cent, the glycosuria was 2.78 and 4.35 per cent. The differences exist likewise if the urine volume and quantitative sugar output are considered. In general, as the diabetes advanced and the dog was subjected to more prolonged hyperglycemia, the sugar threshold rose, but yet with the more severe diabetes there was a more active sugar excretion on February 19 than on November 19.

Dog B2-79, in the stage of severe diabetes over a year after operation, received 1 gm. of glucose per kilo per hour and did not show glycosuria when the plasma sugar had reached 0.230 per cent. In another experiment with 1.5 gm. per kilo per hour, the threshold of disappearance of glycosuria is shown in the table in the preceding paper.[11] There was very faint glycosuria from 3.45 to 4 p.m., while the plasma sugar was falling from 0.264 to 0.208 per cent, and none from 4 to 4.15 with plasma sugar of from 0.208 to 0.226 per cent.

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[11] Allen and Wishart,[1] p. 450.

Carbohydrate Feeding.

The normal dog, No. C3-32, weighing 36 kilos, was given 1 liter of 50 per cent glucose solution by stomach tube. The results are shown in Table I of the ensuing paper.[12] The first trace of glycosuria found in frequent catheterizations came after 30 minutes, and the blood sample taken at this time showed 0.222 per cent of plasma sugar. Thereafter the blood sugar concentration fluctuated (as sometimes happens with large doses and particularly with nausea, due probably to irregularities of absorption), but heavy glycosuria occurred with much lower hyperglycemia, although the blood became concentrated as judged by hemoglobin and corpuscle percentage estimations.

Dog B2-00, partially depancreatized non-diabetic, weighing 14 kilos, received 3 gm. of glucose per kilo in 30 per cent solution by stomach tube. The first trace of glycosuria was obtained with a plasma sugar concentration of 0.192 per cent.

Dog B2-60, partially depancreatized non-diabetic, weighing 45 kilos, received 12 gm. of glucose per kilo of body weight in 40 per cent solution by stomach tube, and the threshold was found to lie between 0.202 and 0.222 per cent.

Dog B2-00, above mentioned, was fed 100 gm. of beef lung, 200 gm. of bread, and 75 gm. of glucose. The threshold of disappearance of glycosuria was determined between 0.154 and 0.149 per cent. The next day the same food was given with increase of glucose to 150 gm. The glycosuria was heavier, and a bare trace was still present after 6 hours with the plasma sugar at 0.128 per cent. 2 days later the same diet was given, and after 6 hours a faint trace of glycosuria persisted with plasma sugar of 0.130 per cent.

Dog B2-60, after removal of more pancreatic tissue but still not quite diabetic, received a feeding test with 100 gm. of lung, 800 gm. of bread, and 200 gm. of glucose, repeated on 4 different days. The threshold of appearance of glycosuria was determined in close agreement in all four tests as lying between 0.200 and 0.222 per cent. This is practically identical with the former test with pure glucose.

Dog B2-00 was fed the above diet of 100 gm. of lung, 200 gm. of bread, and 150 gm. of glucose when in the stage of mild diabetes. The threshold of appearance of glycosuria was determined between 0.145 and 0.152 per cent. 6 weeks later the same diet was fed, after hyperglycemia during most of the interval and some aggravation of the diabetes, and the first trace of glycosuria was found with plasma sugar of 0.204 per cent.

Dog B2-01, in the stage of mild diabetes, received a feeding test of 100 gm. of lung, 200 gm. of bread, and 30 gm. of glucose, repeated four times at 2 day intervals. The threshold of appearance of glycosuria was almost exactly 0.130 per cent on all four occasions, and was thus in keeping with the uniformly low thresholds in the early record of this dog. 3 months later, the diabetes meanwhile having been well controlled by diet, the same test was repeated. The threshold of appearance of glycosuria was 0.135 per cent. The threshold of disappearance

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[12] Allen, F. M., and Wishart, M. B., *J. Biol. Chem.*, 1920, xliii, in press.

was not exactly determined, but a trace of glycosuria remained when the plasma sugar had fallen to 0.128 per cent. The same test was repeated 1 year later, when the diabetes was still under control and glycosuria or hyperglycemia had been permitted only in occasional short experiments during the interval. The threshold of appearance of glycosuria was between 0.141 and 0.147 per cent. The threshold was thus evidently rising. But the naturally low thresholds of this dog find their strongest contrast in the terminal stage with maximal severity of diabetes, when the plasma sugar was 0.322 per cent during fasting without glycosuria.[13]

Dog B2-02 was tested in the stage of mild diabetes after a meal of bread and soup, and glycosuria appeared with a plasma sugar concentration of 0.162 per cent.

Dog B2-88 was tested at a stage when the diabetes was mild but yet more advanced than that of the preceding dogs, so that chronic hyperglycemia was present on mixed diet. The fasting plasma sugar was 0.189 per cent. After a feeding test of 100 gm. of lung and 200 gm. of bread, it rose in successive analyses to 0.257 per cent without glycosuria, and 5 hours after feeding the first faint trace of glycosuria appeared with 0.286 per cent plasma sugar.

Protein Feeding.

As glycosuria from protein was taken as the criterion of severe diabetes, it follows that a renal sugar threshold on protein feeding can be determined only in severe cases. There is a considerable intermediate class of dogs (like a corresponding class of human patients) which have chronic hyperglycemia on protein diets, with no glycosuria even though the plasma sugar is constantly above the threshold of normal individuals. After a protein meal there is often a rise of blood sugar like that following carbohydrate in milder cases. As examples may be mentioned plasma sugar concentrations of 0.204, 0.222, 0.232, and 0.294 per cent in four dogs of this series under these conditions without glycosuria. These values do not establish thresholds, except that it can be said that the thresholds lie above these levels.

Examples of sugar thresholds on protein diets are given incidentally elsewhere, particularly under "Fat feeding." The question whether the protein itself has any influence upon the threshold in severe diabetes cannot receive a clear answer under the conditions, for since

[13] Allen, F. M., *J. Exp. Med.*, 1920, xxxi, 600.

these dogs cannot tolerate adequate protein diets it follows that in any prolonged experiments they must have been subjected to fat feeding, hyperglycemia, or marked undernutrition, any of which may possibly affect the threshold. In short experiments there is evidence that the threshold is approximately the same whether the hyperglycemia is produced by protein or by carbohydrate.

Special Influences Affecting the Threshold.

A. Fasting.—

Dog B2-00, partially depancreatized non-diabetic, as above mentioned under "Subcutaneous glucose injections," received 3 gm. of glucose per kilo and showed slight glycosuria with 0.163 per cent plasma sugar. After the dog had fasted for 1 week the same subcutaneous dose was repeated, and the plasma sugar reached 0.238 per cent without glycosuria.

Dog C3-22, with severe diabetes which had been partially controlled for 6 weeks with diets very high in fat, showed hyperglycemia as high as 0.256 per cent without glycosuria. After 5 days of fasting, a feeding test fixed the threshold of appearance of glycosuria at 0.159 per cent.

The different effects of fasting possibly have some relation to the preceding diet.

B. Excess of Sugar.—Under "Fasting plasma sugars" mention was made of the fact that the high thresholds characteristic of severe diabetes are generally found after long standing hyperglycemia, and the latter is with some reason regarded as a factor in raising the threshold, though some examples were given of high thresholds after briefer hyperglycemia. A normal cat subjected to subcutaneous glucose injections through many months[14] developed a remarkable pathologic condition, particularly with regard to nervous manifestations, and at the close a blood sugar concentration of 0.539 per cent was found without glycosuria. The simple osmotic disturbance of the subcutaneous injections may have been responsible for the high sugar threshold as well as for the other pathological symptoms. Dogs B2-00 and B2-01, frequently mentioned in this and preceding papers,[15] were among numerous animals subjected to the highest possible carbohydrate diets through months and years, without elevation of the

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[14] Allen,[8] pp. 159, 164, 168. Microphotograph of adrenal medulla, Fig. 1.
[15] Allen, F. M., *J. Exp. Med.*, 1920, xxxi, 396; Allen,[9] pp. 562, 564.

threshold before they became diabetic. Allowance must be made for the fact that the hyperglycemia in such animals is generally brief, and is never actually continuous over any long time unless the animal becomes diabetic. A considerable degree and duration of hyperglycemia are occasionally attained in certain dogs barely on the edge of diabetes, as Dog B2-86, previously described.[16] After removal of about $\frac{4}{5}$ of the pancreas on April 7, glycosuria was maintained almost daily by enormous glucose diets. In the feeding test with 400 gm. of bread and 200 gm. of glucose on May 4, the urine taken at frequent intervals first showed sugar about the end of the first $\frac{1}{2}$ hour, with plasma sugar of 0.196 per cent. From the table given for May 13 it may be judged that the threshold on that date was not far different. The threshold therefore seems to be slightly elevated as compared with the normal; but as the dog was old and his threshold before the pancreas operation had not been determined a conclusion is unsafe. Furthermore, the tables referred to furnish evidence that important hyperglycemia must have been present for about a month, yet the threshold was not so high as that of severely diabetic dogs a few days after operation.

C. *Fat Feeding.*—Most of the severely diabetic patients and animals with high sugar thresholds have been on diets containing considerable fat for longer or shorter periods. This factor cannot be excluded in any of the high thresholds of severely diabetic animals mentioned thus far, except in a few cases of fasting. The thresholds have been noticeably high in the animals receiving the highest fat diets, and one of the highest in the series was observed under these conditions.[17] Here the threshold was evidently above 0.4 per cent, and the suggestion was made that excessive fat feeding may affect the kidney function injuriously.

Some indications of a direct influence of fat seemed to be furnished by Dog B2-79. The high thresholds previously mentioned (p. 477) were obtained after very high fat diets (up to 300 gm. of lard or suet daily for over a month). Then for 5 days the only diet was 500 gm. of lung daily without glycosuria. At the end of this time a meal of 1 kilo of lung was given, and glycosuria began when the plasma

[16] Allen,[15] p. 385.
[17] Allen, F. M., *Am. J. Med. Sc.*, 1917, cliii, 348, 352.

sugar reached 0.159 per cent.   The high fat diets were then resumed for 3 days, after which a fasting plasma sugar of 0.200 per cent was found without glycosuria.   After 2 months more of diets consisting predominantly of fat, a protein-feeding test showed a threshold of 0.334 per cent.

Several observations indicate that a single feeding with fat has no demonstrable influence on the sugar threshold.   No prolonged experiments with fat feeding were performed in normal or mildly diabetic animals.

D. Acidosis.—This was one of the factors considered by Fitz[18] in his study of the renal function in diabetes.   It can be excluded in regard to most of the high thresholds and also the fat diets of this series, because the dogs were free from acidosis.   Dog B2-79 had slight acidosis with some of the thresholds mentioned, and Dog C3-56 had more marked acidosis at certain times.   Sugar thresholds were not obtainable in coma cases in dogs because of the continuous glycosuria.

Considerable hyperglycemia without glycosuria may be found in experiments with large intravenous injections of either acetone bodies or mineral acids, such as will be described elsewhere, but the renal changes can scarcely be classified as typical of acidosis because of the chance that they are due merely to this mode of administration of a toxic foreign substance.

E. Cold.—The opportunity was taken to determine approximately the sugar thresholds of several dogs used for experiments with cold to be described elsewhere,[19] but this degree of cold seemed to produce no important alterations as compared with the thresholds of the same dogs at ordinary temperatures.

F. Exercise.—No sugar thresholds were determined in exercise experiments, but the normal dog, No. B2-90, with exercise alone attained a plasma sugar concentration of 0.256 per cent without glycosuria, which is probably higher than the threshold of any normal dog under ordinary conditions.

G. Infections.—A normal dog shortly before death from rabies had a plasma sugar of 0.450 per cent without glycosuria.   Sugar thresh-

[18] Fitz, R., *Arch. Int. Med.*, 1917, xx, 809.
[19] Allen, F. M., Am. J. Physiol., 1921, liv, 425.

olds were not determined in dogs with ordinary infections and would probably have little significance because of the varying grades of renal injury to be expected in such cases.

H. Hemorrhage.—A normal dog weighing 10.8 kilos was bled rapidly from the femoral artery to the amount of 500 cc. The preparation had been made in advance so that there was no pain or struggle. The plasma sugar at the beginning of bleeding was 0.121, at the end 0.238 per cent, without glycosuria. The hyperglycemia of hemorrhage is well known, and the apparently elevated threshold agrees with observations of Wilenko [20] and of Epstein and Baehr.[4]

I. Unilateral Nephrectomy.—

Dog D4-69 in January, 1917, underwent removal of the left kidney, followed by partial pancreatectomy not quite sufficient to produce diabetes. In March, on diets of bread and soup with 200 gm. of glucose, plasma sugar concentrations as high as 0.264 per cent were observed without glycosuria. No reason was known for the apparently elevated threshold except the kidney removal.

J. Epinephrine.—Pollak[21] discovered that repeated injections of epinephrine diminish the renal permeability for sugar.

The normal dog, No. C3-92, weighing 17.6 kilos, on May 16, 1916, received 10 cc. of Parke-Davis adrenalin ($\frac{1}{1,000}$) solution subcutaneously. The threshold of appearance of glycosuria was determined at 0.152 per cent plasma sugar, the threshold of disappearance at 0.118 per cent. On May 23 the same dose was repeated, and the threshold of appearance of glycosuria was approximately 0.380 per cent. The dose was repeated May 29, and the plasma sugar rose to 0.418 per cent without glycosuria. Lard was fed on May 23 and white clay as a control on May 29. Probably neither of these influenced the threshold, for several similar examples could be cited which are free from such factors; but it seems true nevertheless that the diuretic activity and other accessory conditions influence the sugar threshold with epinephrine. Especially in fasting animals plasma sugars as high as 0.278 per cent have been seen with the first dose of epinephrine without glycosuria.

K. Drugs.—Narcotics and other drugs which produce hyperglycemia and glycosuria (supposedly due largely to asphyxia) at the same time impair the renal function for sugar. For example, a normal dog was deeply narcotized with magnesium chloride intramuscularly,

[20] Wilenko, G. G., *Arch. exp. Path. u. Pharmakol.*, 1912, lxviii, 297.
[21] Pollak, L., *Arch. exp. Path. u. Pharmakol.*, 1909, lxi, 376.

and when near death was partially revived with calcium chloride intravenously. A trace of sugar appeared in the urine when the plasma sugar reached 0.500 per cent. Figures almost as high have been found with the huge doses of morphine necessary for deep narcosis of dogs. Examples of such drug action might be multiplied, probably without much significance.

L. *Pancreatic Function, or Severity of Diabetes.*—One of the questions of chief interest was a possible relation between the renal function and the internal pancreatic function. It may be noticed under "Fasting plasma sugars" that the dogs which developed a high renal threshold very early after operation were those which had very small pancreas remnants and correspondingly severe diabetic tendencies. On the other hand, clinical literature contains occasional statements that some cases of diabetes begin with glycosuria with little or no hyperglycemia. To obtain information whether complete lack of the pancreatic function may make the kidneys more or less permeable for sugar, observations of the threshold of appearance of glycosuria were carried out on two totally depancreatized dogs.

Dog C3-01, a collie aged 3 years, thin at a weight of 15 kilos, after 24 hours of fasting before operation had plasma sugar of 0.099 per cent. In the midst of the 'operation, when the entire pancreas had been dissected free except that its duct and principal blood vessels were not yet severed, the plasma sugar was 0.236 per cent without glycosuria. On removal from the table 15 minutes later glycosuria was still absent and remained so for 2¾ hours; then with the appearance of slight glycosuria the plasma sugar was 0.270 per cent. At the end of 5 hours the plasma sugar was still 0.270 per cent, with 1.6 per cent urinary sugar. On the following days, with the usual heavy glycosuria during fasting, the plasma sugars ranged between 0.3 and 0.5 per cent.

Dog C3-60, a mongrel, in medium condition at a weight of 12.5 kilos, had plasma sugar of 0.208 per cent immediately after total pancreatectomy, without glyco- ' suria. The exact threshold was not determined, but 4 hours later there was heavy glycosuria with plasma sugar of 0.300 per cent.

Doubtless the state of nutrition, the anesthesia, and other accidental factors influence the exact threshold and the interval after operation at which glycosuria begins, but these and similar observations seem to exclude any abnormally low renal thresholds at any stage following total pancreatectomy.

DISCUSSION.

At least three groups of causes may be imagined for the alterations of renal function in diabetes; namely, (*a*) direct renal and vascular injuries, (*b*) general metabolic conditions, and (*c*) influences associated specifically with the diabetes.

(*a*) Clinical diabetes is not infrequently associated with some degree of nephritis or arteriosclerosis, and even where these are not evident there is a possibility that the same infection or intoxication which damaged the pancreas may have left some anatomic or functional injury in the kidney. Animal experiments offer the opportunity either of studying diabetes without these possible complications, or of producing different forms of renal injury for investigation. Though the supposition of an adrenal element in diabetes is opposed by weighty facts and supported by none, the use of epinephrine as a drug gives an interesting illustration of a toxic renal or vascular alteration. When the acute effects have subsided, it is questionable whether the finest study can reveal any anatomic changes, and the animals are certainly free from albuminuria and the ordinary clinical symptoms of nephritis. Yet there is an elevation of the sugar threshold which is far greater than can be accounted for by the brief hyperglycemia, and which is either permanent or at least of considerable duration. Animals which have undergone removal of $\frac{1}{2}$ to $\frac{3}{4}$ of their total renal tissue offer some points of interest not only for the sugar threshold in the old sense but also for their sugar excretion by Benedict's new method, for determining to what extent the latter represents general carbohydrate metabolism or merely a function of the kidney itself.

(*b*) Some general metabolic alterations might consist in accidentally associated endocrine disorders, which, notably in the case of the hypophysis, are recognized as affecting renal function. The nutritive state is another possible factor. The so called famine or war edema is presumably similar to that of many diabetic cases. The latter is usually accompanied by a high renal threshold for both sugar and salt, but the relation is not known to be invariable; equally high thresholds are found in cases without edema, and the etiology may be independent.

Alterations in blood volume are a factor adequately discussed by authors already quoted. A possibility not much considered heretofore is an influence of food upon renal function. Barrenscheen[22] claimed that an "oatmeal cure" may diminish the permeability of the kidneys for lactose, though the fact itself and the interpretation, whether due to carbohydrate, salt, or something else, may bear further investigation. On the other hand, there is abundant evidence that in the declining stage of a glycosuria there is ordinarily a lowering of the renal threshold, in the sense that sugar continues to pass into the urine at a level of plasma sugar concentration distinctly lower than that necessary to produce glycosuria in the first place. One hypothetical explanation may be suggested by the well known Armanni or Ehrlich phenomenon of glycogen deposition in certain segments of the renal tubules. If these deposits be accepted as representing sugar absorption by the tubule cells, it is conceivable that cells thus saturated may either continue for a time to discharge sugar, or (more probably) that they may fail to resorb sugar as actively as usual from the glomerular filtrate, and thus the prolongation of glycosuria might be explained under either of the theories of renal secretion. A possible influence of fat feeding is also suggested by other evidence. Chemical analyses in the literature indicate that the kidney is one of the organs in which fat is deposited during lipemia. Especially with heavy lipemia this can be confirmed microscopically, and it is interesting that the cells which stuff themselves with fat are the same ones which are filled with glycogen in glycosuria. The two may be combined; carmine stains then show the glycogen granules to be distributed around, not in, the vacuoles, and the latter are shown by Sudan stains to be fat. This fat infiltration accords with the traces of fat found in the urine with heavy lipemia, and can be interpreted according to either theory of renal secretion. If fat feeding causes a diminution of renal permeability for sugar, it may be associated in some unknown way with these morphologic changes or due to invisible alterations in cell membranes.

(c) One condition in diabetes which may affect the kidneys is the acidosis. This supposition is supported by the well known albuminuria and showers of casts appearing as precursors of coma. This can,

[22] Barrenscheen, H. K., *Biochem. Z.*, 1912, xxxix, 232.

however, be easily excluded as a sole cause by the fact that dogs and most human patients studied in sufficiently early stages show a considerable elevation of the sugar threshold without acidosis. The typical onset of diabetes in all but the most acute cases seems to be by a gradual rise of blood sugar, frequently traceable through months in both animals and patients, before glycosuria appears; there is generally continuous hyperglycemia in the next stage of occasional or "alimentary" glycosuria, and generally next a period of continuous glycosuria without acidosis. In most cases at any of these stages the sugar threshold is found to be already high.

This elevation of the threshold may be attributed to the prolonged hyperglycemia itself, a common notion being that the kidney by habituation comes to "tolerate" glucose. This explanation may contain much truth, but yet it appears that a high threshold may develop very early in severe diabetes and may be absent after more intense and prolonged hyperglycemia and glycosuria in normal or mildly diabetic animals. Also, if this elevation is a mere habituation, it might be expected to be lost after removal of the cause, but in the writers' experience severely diabetic patients and animals have shown characteristically high sugar thresholds after a year or more of continuously normal blood sugar. The reason for the low thresholds of exceptional clinical cases is also not clear.

A specific relation of the renal and pancreatic functions is not demonstrable in the sense of any increased readiness of sugar excretion following total pancreatectomy. An opposite view is sometimes entertained, that the elevated sugar threshold is to be regarded as a protective mechanism for saving sugar to the body. It might further be considered plausible that this device is more needed and called more into play with increasing severity of the diabetes. There is no real disproof of this assumption. It is true furthermore that some extra utilization of carbohydrate is probably forced by any obstruction of excretion, according to the principle of the metabolism of plethora, as long as any appreciable power of utilization is retained by the organism. It was thus found in the preceding paper that renal impermeability confuses judgment of the urinary findings in glucose tolerance tests, particularly intravenous tests of short duration. It has less influence upon feeding tests or any tests of long duration,

because the blood sugar rises high enough to cause heavy glycosuria even at the higher threshold. Theoretically the interpretation of the elevated threshold as a teleologic adaptation is an unproved and improbable assumption. The chief error which can be refuted is the supposition that the renal impermeability is of any real benefit to the diabetic organism in a practical sense. This involves the usual confusion between diabetes and glycosuria), and is closely allied to the discredited clinical practice of giving arsenic, uranium, and other drugs which perhaps to some extent suppress glycosuria by injuring the kidneys. Two important principles, which are perfectly clear in animal experiments, are equally applicable clinically. First, diabetes is deficiency of the internal secretion of the pancreas, and this deficiency cannot be compensated by any mere blocking of renal excretion. An extreme illustration is in totally depancreatized dogs, which die all the more quickly when their sugar is dammed back by nephrectomy or ureteral ligation. Second, the essential trouble to be combated in diabetes is not the mere loss of food material in the urine. The saving by renal impermeability, and also the metabolism of plethora induced by increased sugar concentration in the blood, can readily be equalled in the milder forms of diabetes by increasing the diet, and as the latter process is harmful the former may be expected to prove so likewise. In severe diabetes the damming back of metabolic products which cannot be utilized is a still more serious complication, as above mentioned. Some clinical illustrations of the above statements are found in the symptoms which occurred with trivial glycosuria and plasma sugar of 0.73 per cent in a patient with combined diabetes and nephritis,[23] and the occurrence of typical diabetic symptoms and complications in patients with hyperglycemia with little or no glycosuria because of a high renal threshold.[24]

The chief criticism of the results contained in this communication is that they possess only the suggestiveness which is more proper to clinical observations, and could not be followed up with conclusive methods and controls to establish causes and laws in the manner to

[23] Allen, F. M., Stillman, E., and Fitz, R., Total dietary regulation in the treatment of diabetes, Monograph of The Rockefeller Institute for Medical Research. No. 11, New York, 1919, Case 61, 413.

[24] Mitchell, J. W., Am. J. Med. Sc., in press.

be expected from animal experiments. The following deductions are possible concerning some of the points chiefly in view.

CONCLUSIONS.

1. A diminished renal permeability for glucose, in the sense of a raised threshold according to tests with Benedict's copper solution, is definitely proved as the rule in diabetic animals. As the same rule holds for the great majority of human diabetics, this demonstration furnishes one more point of similarity between the experimental and clinical conditions.

2. Various possible causes for the elevation of the threshold are discussed. The prolonged excess of sugar in the blood may be an important factor, but some considerations seem to oppose it. Certain observations suggest that high fat diets may raise the sugar threshold in diabetes even without acidosis. The renal function may be affected by various extraneous causes, of which the elevation of the threshold by epinephrine is one interesting example.

3. No interrelation of the renal and pancreatic functions is demonstrable in the sense of an increased readiness of sugar excretion even in totally depancreatized animals. An elevation of the threshold seems to be connected particularly with severity of the diabetes, but a teleological interpretation of this as a protective mechanism for saving sugar to the body is considered improbable.

A MODIFICATION OF VAN LEERSUM'S BLOODLESS METHOD FOR RECORDING BLOOD PRESSURES IN ANIMALS.

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PLATES 25 TO 28.

A satisfactory method does not exist for taking the blood pressure in laboratory animals so that observations can be repeated on successive days over long periods of time. The advantages of possessing such a method are obvious. A single animal may behave differently on different occasions; it may react differently to different members of the same group of agents. A method such as the one described in this communication affords an opportunity for ascertaining the usual behavior of an animal.

The essential part of the method was described in 1911 by Van Leersum[1] and was utilized by him for studies on rabbits. It consists in making the carotid artery accessible to direct examination. In order to do this an aseptic operation under ether anesthesia is performed. Two longitudinal and parallel incisions are made in the neck of the animals (dogs were utilized by us) about 7 to 10 cm. long (Text-fig. 1), the distance between the two being about 3 to 4 cm. The stretch of skin between the two incisions is freed from the underlying muscle. As much of the subcutaneous tissues is permitted to remain attached to the skin as will insure to it a sufficient blood supply. The carotid artery is then found and gently freed of· the other structures contained in the carotid sheath; when possible a generous amount of areolar tissue is permitted to continue to surround the artery. The length of the artery freed in this manner should be

[1] Van Leersum, E. C., Eine Methode zur Erleichterung der Blutdruckmessung bei Tieren, *Arch. ges. Physiol.*, 1911, cxlii, 377.

several centimeters longer than the skin incisions. The edges (Text-fig. 2) of the stretch of skin are next sewed together so that the skin surrounds the artery as a tube. The outer edges of the two incisions are then approximated and sutured to restore the skin of the neck. The adjustment of the edges at the two ends of the tube and of the skin of the neck must be exact. The ligature material may be silk, but we have found retaining sutures of chromic gut and intervening
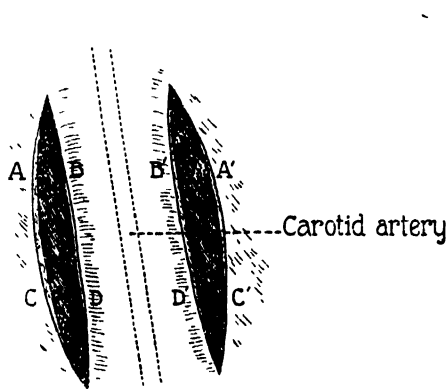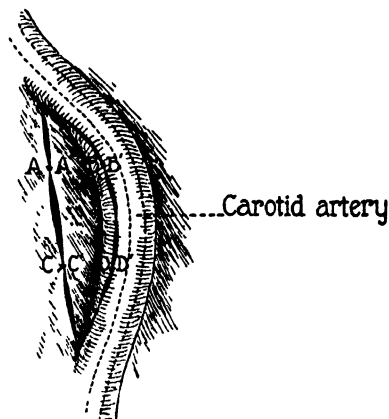
TEXT-FIG. 1.   TEXT-FIG. 2.

TEXT-FIG. 1. Two vertical and parallel incisions in the neck are shown. The position of the carotid artery in respect to the stretch of skin between them is indicated.

TEXT-FIG. 2. The formation of the tube of skin containing the carotid artery is shown. After freeing the artery from the carotid sheath it is lifted out and surrounded by the stretch of skin between the incisions shown in Text-fig. 1. The operation is completed by suturing the points BB', DD' and AA', CC'.

sutures of plain gut more satisfactory. A dry dressing is interposed between the carotid tube and the repaired neck. The wound is dressed with dry gauze and the bandage is held in place with adhesive plaster. The wound may be dressed after an interval of 4 to 6 days. The sutures are removed when sufficient healing has taken place. The wound should heal by primary intention (Fig. 1).

The carotid artery surrounded by its tube of skin is now accessible to examination. In the technique employed by Van Leersum it is
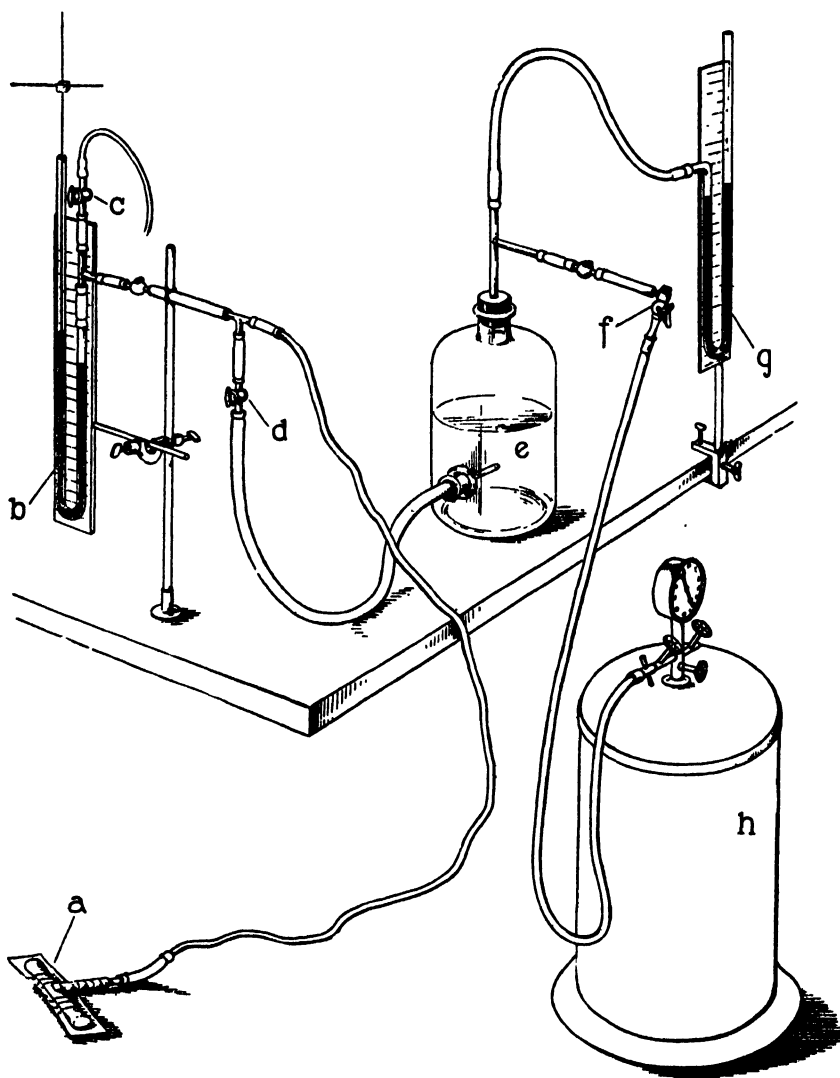
TEXT-FIG. 3. Diagram of the cuff system. *a*, the cuff for surrounding the carotid tube. *b*, mercury manometer and writing point. *c*, valve permitting escape of pressure from the system. *d*, valve for elevating pressure within the system. *e*, water bottle kept at a pressure of about 250 mm. of mercury (*g*), *f*, escape valve. *h*, air pressure tank to supply pressure for *e*.

surrounded by a rubber cuff designed like that of von Recklinghausen but of smaller dimensions, 1 or 2 cm. wide by 3 or 4 cm. long. The pressure in the cuff is elevated until pulsation is no longer felt in the artery distal to the cuff. This pressure is read on a mercury manometer.

We have introduced a graphic method of recording pressure. The system between cuff and manometer is filled with water (Text-fig. 3). Except where it was impossible, we have used lead tubing. Pressure is introduced through a cock (d) into the system from a bottle kept at a pressure of about 250 mm. of mercury. It is allowed to escape through a stop-cock (c) at the proximal limb of the manometer.

By means of these cocks pressure can be elevated or can be permitted to fall either stepwise (Fig. 2) or gradually (Figs. 3 and 4). The record is made on smoked paper by a writing point supported on a float on the mercury column. The base-line is inscribed. Minimum and maximum oscillations utilized by Erlanger as the points of maximum and minimum pressures may be utilized.[2] We have obtained deflections of sufficient size to permit us to make accurate observations. If it is desirable, it is possible to train dogs to lie quite still for an hour or more without anesthesia or narcotics. To be able to do this has obvious advantages.

We are now making use of the method for the study of drugs of the digitalis series in which we desire to obtain simultaneous blood pressure records and electrocardiograms. The results of these studies will be given in another communication.

[2] To avoid the oscillations due to the shock of the impinging blood stream on the cuff, a second cuff distal to the first may be introduced about the carotid tube. The pressure of this cuff is maintained at the diastolic level. Only when the pressure in the artery is high enough to pass the proximal cuff, will the distal one be affected and a record be inscribed.

EXPLANATION OF PLATES.

PLATE 25.

FIG. 1. The carotid tube of one of the dogs after recovery.

PLATE 26.

FIG. 2. Below is the base-line. The blood pressure is inscribed by the method of intermittent escape of pressure. The systolic pressure is recorded at the step below the top (202 mm. of mercury); diastolic pressure at 100 mm.

PLATE 27.

FIG. 3. Record of blood pressure taken by the method of gradual escape. The systolic pressure is 154 mm. of mercury; the diastolic pressure is 88 mm. The top line is a signal to indicate when an electrocardiogram was made. The bottom is the base-line.

PLATE 28.

FIG. 4. Record of blood pressure taken by the method of gradual increase of pressure. The systolic pressure is 160 mm. of mercury; the diastolic pressure is 88 mm. The top line is a signal to indicate when an electrocardiogram was made. The bottom is the base-line.

FIG. 1.
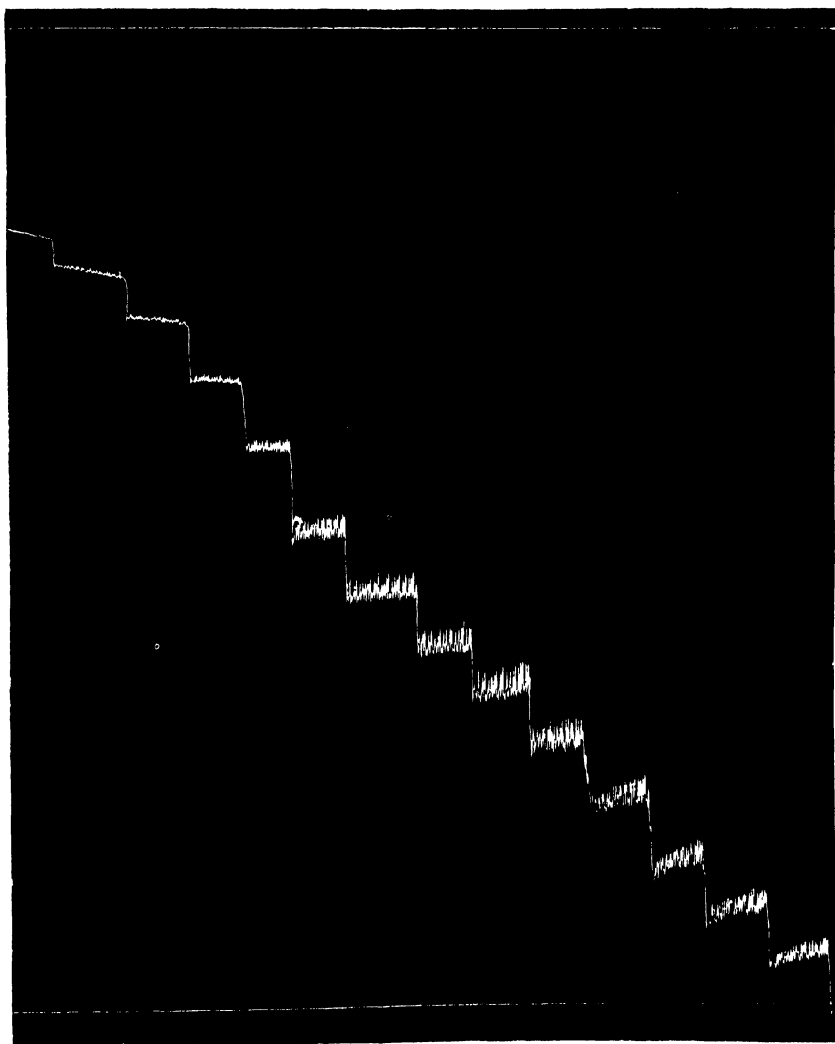
FIG. 2.
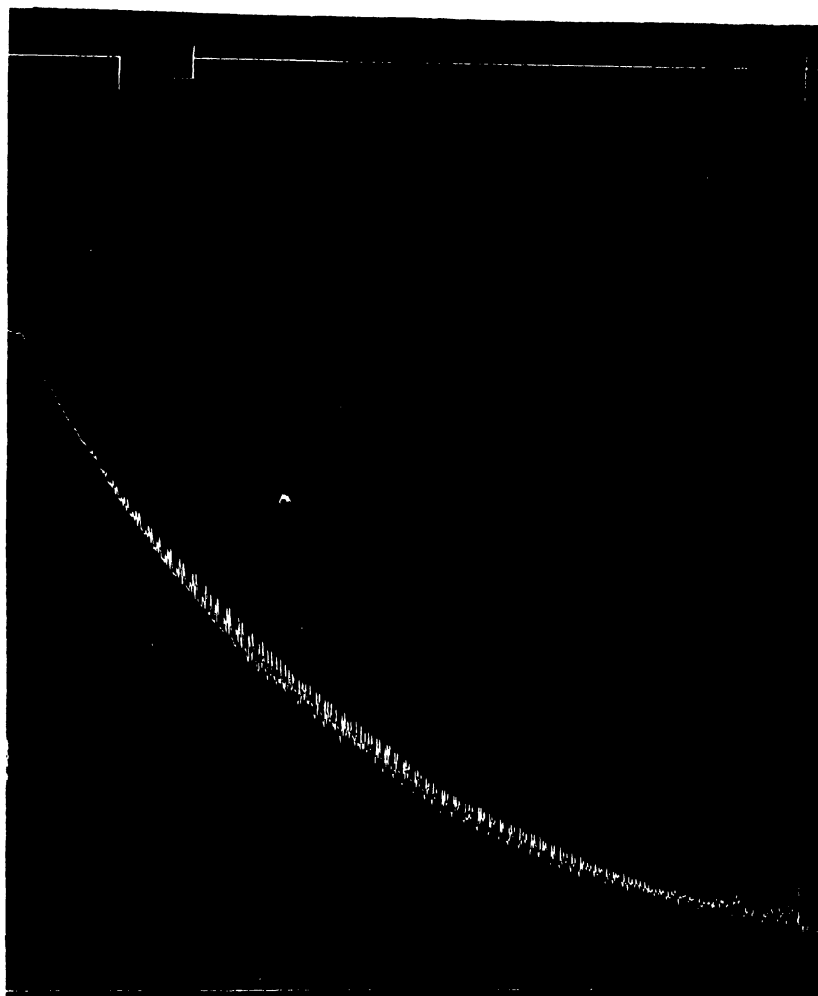
Fig. 3.
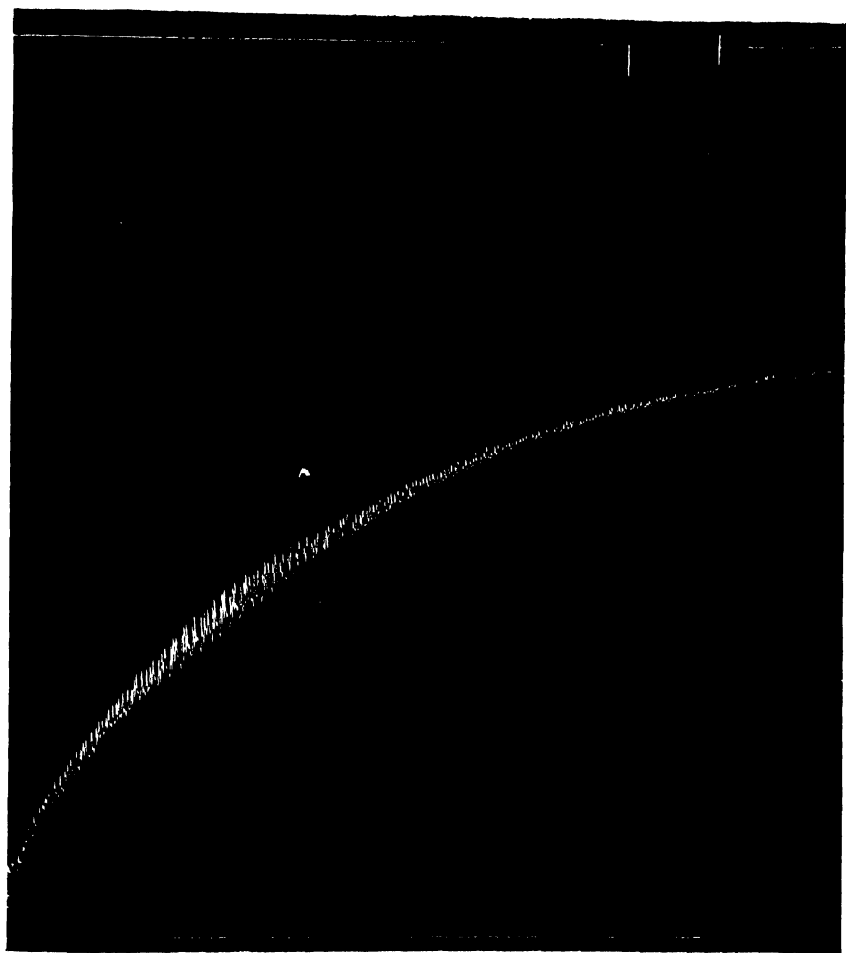
FIG. 4.

A GROUP OF PARATYPHOID BACILLI FROM ANIMALS CLOSELY RESEMBLING THOSE FOUND IN MAN.

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In a previous paper (1) the writer described a group of paratyphoid bacilli, isolated from animals, that with the usual cultural methods seemed to be the same as paratyphoid B, or *Bacillus schottmülleri*, as Winslow, Kligler, and Rothberg (2) suggest we call the latter organism. Serologically they could be separated from one another by agglutination absorption tests and to a certain extent by the type of clumps formed in immune sera. Five new cultures belonging to this group have recently been studied and a further effort has been made to differentiate them from the human paratyphoids by a method less complicated than agglutination absorption.

Five of the cultures studied came from swine infected with hog-cholera virus, two came from guinea pigs, and one each from a child, a cow, a pigeon, and a mouse. Four cultures of *Bacillus schottmülleri* obtained from Dr. C. Krumwiede were used for comparison. The date of isolation and other facts regarding the above cultures are given in the paper already referred to. In addition, cultures isolated by Dr. Smith in 1918 from five calves have been studied and found to belong to the same group, and still more recently additional cultures of this group have been isolated from swine infected with hog-cholera virus. It is evident that these organisms are quite widespread among our domestic animals but just what relation they have to animal diseases will require much further study. In hog-cholera they may tend to emphasize the intestinal lesions, but cases of this disease occur with extensive intestinal changes from which organisms of this group cannot be isolated.

Cultural Studies.

Sixteen cultures of the animal paratyphoids have been studied culturally and they have all corresponded to one another and to *Bacillus schottmülleri*. The results of the study are given in Table I, which includes the characters of *Bacillus enteritidis* and of the hog-cholera bacillus.

Some of the strains were tested with inosite with unsatisfactory results as the amount of acid produced by the paratyphoids was so small that it seemed possible that it was due to impurities. Other carbohydrates might have been included in the study but they are so

TABLE I.

Cultural Characters of Paratyphoids Studied.

Organisms.	Motility.	Gram stain.	Indole.	Hydrogen sulfide.	Gelatin liquefied.	Dextrose.	Maltose.	Mannitol.	Xylose.	Dulcite.	Arabinose.	Lactose.	Saccharose.	Salicin.	Glycerol.	Raffinose.
B. *schottmülleri* (paratyphoid B)	+	−	0	+	0	AG*	AG	AG	AG	AG	AG	0	0	0	0	0
B. *enteritidis*	+	−	0	+	0	"	"	"	"	"	"	0	0	0	0	0
Animal paratyphoids	+	−	0	+	0	"	"	"	"	"	"	0	0	0	0	0
Hog-cholera bacilli	+	−	0	0	0	"	"	"	"	0	0	0	0	0	Slightly acid.	0

* AG indicates acid and gas formation.

expensive that they would be of no practical value in differentiating the two groups. As shown in the table, no cultural differences have been found between *Bacillus enteritidis*, *Bacillus schottmülleri*, and the animal paratyphoids.

In the hope that a difference might be detected between animal and human paratyphoids, by a study of the hydrogen ion concentration, cultures were made in dextrose and xylose broth. Both groups gave a hydrogen ion concentration of pH 5.0 in dextrose broth after 4 days incubation, while in xylose broth the pH varied from 5.6 to 5.2. Some of the animal strains acted more slowly and others apparently utilized more of the carbohydrate than did the human strains. The difference is so slight, however, that it is of no value in classification.

Serological Studies.

When living cultures are used for immunization of rabbits the sera produced will usually agglutinate both the human and animal paratyphoids to the same titer limit. At times, however, one gets a serum that will agglutinate bacilli of the group used in the immunization in higher dilutions than it does those of the other group. This is apparently due to the individual rabbit rather than the bacteria, for the same culture will act differently in different rabbits.

Cultures from both human and animal sources are agglutinated in only the lower dilutions of sera from animals immune to *Bacillus enteritidis* and as the results obtained in the present study do not differ from those previously given they need not be repeated here.

When 24 hour bouillon cultures are used as antigens the clumps formed by bacilli of the same group as the immunizing strain are flocculent and after 2 hours incubation form a mass occupying from one-quarter to one-half the column of liquid. Bacilli of the other group as a rule give very compact clumps which after standing in the refrigerator over night form a thin film on the bottom of the test-tube. This difference in the clumping is striking but unfortunately it does not always hold true. If growth is more abundant than the average, flocculent clumping may occur where a compact type is expected. The amount of dextrose in the bouillon influences the type of clumps formed. Apparently the amount of growth rather than the change in reaction is responsible for the change. As a rule, however, one can differentiate fairly well between the two groups by the type of clumps, but it can only be regarded as a tentative means of differentiation.

When heated or formalinized bouillon cultures or suspensions in salt solution of the growth from agar slants are used as antigens, agglutination will occur in the same dilutions of serum and the clumps are so nearly alike in character that the two groups cannot be differentiated.

When formalinized bouillon cultures are standardized and agglutinated according to Dreyer's method (3) with the readings made after 2 hours incubation at 50°C. in the water bath and 15 minutes at room temperature, the two groups cannot be differentiated by the degree of agglutination or the type of clumps.

Numerous agglutination absorption tests have been made and the results have confirmed those previously reported. The animal strains will absorb from *Bacillus schottmülleri* sera the agglutinin for all the

TABLE II.

Summary of Absorption Experiments with Serum of Rabbit 9, Immunized to Calf-Typhus V by Injection of Living Cultures.

Culture tested.	Titer limit of serum saturated with.					
	Nothing; i.e., control.	B. schott-mülleri 232.	B. schott-mülleri 242.	Calf-typhus V.	Swine-typhus I.	Swine-typhus V.
Calf-typhus I...............	51,200	25,600	25,600	—	—	—
" III............	51,200	—	—	—	400	400
" IV............	51,200	25,600	25,600	800	—	—
" V...............	51,200	25,600	25,600	200	400	400
Swine-typhus I.............	51,200	12,800	12,800	—	200	—
" V.............	51,200	25,600	25,600	—	—	200
Pigeon-typhus...............	51,200	25,600	25,600	—	—	—
Mouse-typhus I.............	51,200	25,600	25,600	—	—	—
Hog-cholera XII............	51,200	400	200	—	—	—
" XIII............	51,200	400	200	—	—	—
B. schottmülleri 232..........	51,200	400	200	100	—	—
" " 242...........	51,200	400	400	—	—	—
" ' " 225...........	51,200	200	800	100	—	—

TABLE III.

Absorption of Agglutinins from B. schottmülleri Serum by Calf-Typhus Cultures.

Culture tested.	Titer limit of serum of Rabbit 10, immune to B. schottmülleri 232, absorbed with.		
	Nothing; i.e., control.	Calf-typhus V.	Calf-typhus I.
Calf-typhus I.............................	12,800	200	400
" IV.............................	12,800	100	100
" V.............................	12,800	200	400
Swine-typhus I...........................	12,800	200	400
B. schottmülleri 225.......................	25,600	25,600	25,600
" " 232.......................	25,600	25,600	25,600

animal cultures without removing those for the human cultures, and, *vice versa, Bacillus schottmülleri* will remove from the sera of animals immune to the animal cultures the agglutinin for the human cultures

and will not remove those for the animal strains. The calf and swine cultures are identical in this respect as is shown in Tables II and III.

While the great majority of the agglutination tests was made with sera of animals immunized by the injection of living bouillon cultures, the possibility that sera produced by the injection of heated cultures might differentiate the two groups has also been considered. The serum of an animal immunized by three injections of a suspension of agar slant growth, killed by heating to 60°C. for 1 hour, agglutinated both groups of bacilli to the same degree. When sera of animals immunized by bacilli heated to 70° for 1 hour were tested, it was found that the bacilli of the immunizing group were agglutinated in a somewhat higher dilution than were those of the other group. The difference was not great enough to make it a valuable means of differentiation. Better results might be obtained by using heated and washed bacilli, but this has not been tried.

Since the type of clumping indicates that the difference in these two groups lies in the flagella, antigens were prepared by shaking suspensions of the bacilli for a short time, centrifugalizing, and using the supernatant fluid for precipitation and complement fixation tests. When the antigen belonged to the same group as the immune serum, the precipitate was more flocculent than when it belonged to the other group, but the amount of precipitation was about the same. When such antigens were used for complement fixation, the inhibition of hemolysis was the same with sera of rabbits immune to either group. Formalinized bouillon cultures used as antigens in complement fixation tests were likewise of no value in differentiating the two groups. These results agree with those of the previous paper where extracts of the bacilli were used as antigens.

The results of the serological tests show that the animal paratyphoids which are usually agglutinated to the titer limit in sera of animals immune to *Bacillus schottmülleri* can best be separated from the latter by agglutination absorption tests. With the methods commonly used in agglutination tests they would be classed as *Bacillus schottmülleri*.

Cross-Immunization Tests.

In the previous paper it was noted that the injection of living cultures of the swine paratyphoids immunized rabbits to a virulent hog-cholera bacillus, whereas rabbits treated in the same way with living cultures of human paratyphoid were not immune. This seemed at that time to indicate that the swine cultures were more closely related to, or possibly were a variety of, the hog-cholera bacillus. These tests have been repeated and the results are given in Table IV.

It will be seen that the calf cultures also immunize rabbits to the hog-cholera bacillus, whereas the human cultures do not. Examination of the records shows that the injection of the animal cultures produces a more severe type of reaction than does the injection of the human cultures. The local lesion is larger and the rise in temperature following a subcutaneous reaction is higher.

Two rabbits were each given a subcutaneous injection of 0.1 cc. of a 24 hour bouillon culture of two strains of human paratyphoid and were chloroformed 1 week later. There was a slight local lesion from which the organisms injected were cultured. The spleen and other organs were normal and cultures from as much as 0.5 cc. of blood, a piece of liver as large as a pea, and stab cultures from the spleen were sterile. Pea-sized bits of spleen from one rabbit showed no organisms, while from the other there was a growth which proved to be due to the organism injected. 1 week after an intravenous injection of 0.1 cc. of 24 hour bouillon cultures, two other rabbits were chloroformed and their spleens were found to be slightly enlarged. Cultures from the spleen of one of these rabbits showed a growth due to the organism injected, whereas the spleen of the other animal failed to show such organism. The blood, liver, and bile of both rabbits were sterile.

These results show that there is very little growth of the human paratyphoid bacilli in rabbits when they are used in the same amounts that will produce a general invasion by the animal cultures. It therefore seems probable that the immunity to the hog-cholera bacillus produced by the animal paratyphoids is due to the fact that they multiply in the body and increase the resistance enough so that the animals are able to withstand the hog-cholera bacilli injected, when the amount of the latter used is about ten times the minimal lethal dose.

TABLE IV.

Test of Power of Various Paratyphoids to Immunize Rabbits to a Virulent Hog-Cholera Bacillus Culture.

Rabbit No.	Inoculated with.	1st injection.	2nd injection.	3rd injection.	Weight Nov. 18.	Result of subcutaneous injection of 0.000001 cc. of 24 hr. bouillon culture of Hog-cholera XII. Rabbit series, Nov. 18, 1919.
		1919	*1919*	*1919*	*gm.*	
1	Swine-typhus II, bouillon culture.	Oct. 4. 0.1 cc. subcutaneously.	Oct. 25. 0.01 cc. intravenously.	—	2,523	Lived.
2	Swine-typhus IV, bouillon culture.	Oct. 4. 0.1 cc. subcutaneously.	Oct. 25. 0.01 cc. intravenously.	—	2,700	"
3	Calf-typhus I, bouillon culture.	Oct. 4. 0.1 cc. subcutaneously.	Oct. 25. 0.01 cc. intravenously.	—	2,164	"
4	Calf-typhus III, bouillon culture.	Oct. 4. 0.1 cc. subcutaneously.	Oct. 25. 0.01 cc. intravenously.	—	2,684	"
5	*B. schottmülleri* 232, bouillon culture.	Oct. 4. 0.1 cc. subcutaneously.	Oct. 25. 0.01 cc. intravenously.	—	2,509	Death in 7 days.
6	*B. schottmülleri,* 242, bouillon culture.	Oct. 4. 0.1 cc. subcutaneously.	Oct. 25 0.01 cc. intravenously.	—	2,561	" " 10 "
7 Control.	Calf-typhus III, suspension of agar growth, heated 1 hr. at 70°.	Oct. 17. 0.5 cc. subcutaneously.	Oct. 24. 1 cc. subcutaneously.	Nov. 1. 2 cc. subcutaneously.	2,520	" " 9 "
8 Control.	Swine-typhus II, suspension of agar growth, heated 1 hr. at 70°.	Oct. 17. 0.5 cc. subcutaneously.	Oct. 24. 1 cc. subcutaneously.	Nov. 1. 2 cc. subcutaneously.	2,752	" " 6 "
9 Control.	No previous treatment.	—	—	—	2,453	" " 8 "

The intimate relation of these organisms is shown by cross-immunization tests made with mice. These animals were immunized at the same time by subcutaneous injections of 0.0001 cc. followed by intraperitoneal injections of 0.005 cc. of 24 hour bouillon cultures. After they had recovered from the latter injection their immunity towards other cultures was tested by intraperitoneal injections of 0.005 cc. of 24 hour bouillon cultures. The results of the test are given in Table V and show that the animal and human cultures immunize against one another and that the calf culture immunized against the swine and *vice versa*.

TABLE V.

*Test of Cross-Immunity of Mice to Paratyphoids of Other Animals.**

Mice immune to.	Result of intraperitoneal injection of 0.005 cc. of 24 hr. bouillon culture.					
	Calf-typhus V.		*B. schottmülleri* 232.		Swine-typhus V.	
	Lived.	Died.	Lived.	Died.	Lived.	Died.
Calf-typhus V..............	4	0	4	0	4	0
B. schottmülleri 232..........	3	1	4	0	4	0
Swine-typhus V............	3	0	3	0	3	0
Controls. No previous treatment....................	0	4	1	3	0	4

* See text for methods of immunization.

DISCUSSION.

The question arises whether we should place these animal paratyphoids, that culturally are the same as *Bacillus schottmülleri*, in a separate group because they fail to absorb the agglutinins from the serum of animals immune to the latter. This failure to absorb agglutinins seems to be a very fundamental difference and these organisms should be regarded as a distinct variety of paratyphoid. No differences either cultural or serological have been detected between the strains derived from swine, calves, and the few strains from the other species that have been studied.

A common name is desirable for this group of organisms and if it is found, upon further study, that these organisms are the same as the

Aertrycke bacillus, isolated by de Nobele (4) from an outbreak of food poisoning, the name of *Bacillus aertryckei* would be appropriate. It is possible that there is one host that harbors these organisms and from it the other animals become infected. If this should prove to be the case, a name indicating this host would be the logical one. On the other hand, the naming of the animal from which a particular culture is isolated is desirable and for the present I propose that they be called typhus with a prefix denoting the animal from which they were isolated. The name typhus has only its long use to commend it, as the organisms are not like *Bacillus typhosus*, nor do they produce a disease that very closely resembles typhoid fever. Another objection is that in the cultures from the smaller animals the word typhus has been used in connection with the paratyphoid disease that is so common. In my experience most of these cultures from mice and guinea pigs belong to the *enteritidis* group and can be separated by their specific agglutination characters, though organisms of the group under consideration also occur. *Bacillus enteritidis* also occurs in the larger animals. I have found it in one pig, Jensen (5), Meyer, Traum, and Roadhouse (6), and others have found it in calves with diarrhea, and Graham, Reynolds, and Hill (7) have found it in an acute disease of horses. In the future it would be well to call these *Bacillus enteritidis* and reserve the use of mouse-typhus, guinea pig-typhus, etc., for the organisms that we have considered in this paper.

It is evident that the group that has been considered in this paper has been encountered before. I have already (1) pointed out that many of the strains of so called *Bacillus suipestifer* probably belong to this group. Bock (8) noted that when he saturated the sera of animals immune to mouse-typhus, *Bacillus suipestifer*, or bacilli obtained from outbreaks of food poisoning with *Bacillus schottmülleri* the agglutinins for the last bacillus were removed while those for the first three organisms were not affected. Sobernheim and Seligmann (9) in studying paratyphoid bacilli noted that three cultures classed as *Bacillus schottmülleri* formed fine clumps in the serum of an animal immune to *Bacillus schottmülleri*. They immunized a rabbit to one of these strains heated to 70°C. and found that the serum agglutinated the three cultures to the same degree while the other *Bacillus schottmülleri* cultures were agglutinated in only the lower dilutions of serum. Especially significant

are the observations of Bainbridge and O'Brien (10). They compared cultures from cases of food poisoning with those from undoubted cases of paratyphoid fever. In *Bacillus schottmülleri* sera the former produced fine clumps and did not absorb the agglutinins for the immunizing strains, whereas the latter formed flocculent clumps and absorbed all the agglutinins from the serum. Their control cultures of *Bacillus suipestifer* acted the same as those from food poisonings, but as these controls were obtained from German laboratories it is probable that they were the same as those I have called swine-typhus. Krumwiede, Valentine, and Kohn (11) separated from *Bacillus schottmülleri* by absorption tests a number of organisms obtained from rodents which probably belong to this group.

As noted above, when suspensions of agar slant growth or killed cultures are used as antigens in agglutination tests the difference between these animal cultures and *Bacillus schottmülleri* could not be detected and it seems possible that the so called paratyphoid B bacilli that Jensen (5), Christiansen (12), and others have associated with diarrhea in calves are the same as the organisms I have called calf-typhus. Many of the paratyphoid B bacilli isolated from food poisonings quite possibly belong to the same group but from the literature one cannot draw any conclusions as the diagnosis has usually been made on the agglutination test without supplementary absorption tests. This subject is important because we want to know what type of infections man gets from the lower animals.

There are several well recognized groups of pathogenic animal paratyphoids besides *enteritidis* and the group considered here. The hog-cholera bacillus, or better, *Bacillus choleræ suis*, described by Smith (13), differs from the others by being highly pathogenic for rabbits. More recently (1) it has been shown to have distinct cultural differences from *Bacillus schottmülleri*. The Voldagsen bacillus described by Dammann and Stedefeder (14) and the "Ferkel typhus" bacillus described by Glässer (15) seem to be identical. They are differentiated from the other paratyphoids by their failure to act on mannitol and the fact that they produce little or no gas. *Bacillus abortus equi*, first described by Smith and Kilborne (16) and later studied by Meyer and Boerner (17) and others, resembles *Bacillus schottmülleri* culturally, except that it fails to produce hydrogen sulfide, and on agar forms a

dry brittle growth. Serologically Meyer and Boerner, and Murray (18) place it in a group by itself. In its virulence for rabbits Smith and Kilborne pointed out that it resembled a mildly virulent hog-cholera bacillus.

Jordan (19) and Reerstorp (20) have found a variety of so called intermediate paratyphoids in the intestinal tract of normal swine, and from children Lewis (21) and others have obtained paratyphoids which have been classified culturally by Graham-Smith (22). It is difficult to determine just what relation these paratyphoids of the normal digestive tract bear to the established groups. They differ serologically and culturally from the members of these several groups but it is conceivable that under certain conditions they might invade the body and change their characters.

Smith and Reagh (23) discussed the possibility of the host changing the agglutinative characters of organisms and such a possibility should be considered here. After passing from animal to man and becoming adapted to the latter, it is quite possible that organisms might change both their cultural and agglutinative characters. Careful study of food poisoning outbreaks due to eating meat containing these animal paratyphoids might throw some light on this subject. If such a change does occur it would result in much confusion. I have from time to time modified slightly the cultural characters of some of these paratyphoids by passage through animals, but on the whole the cultural and especially the agglutination characters are remarkably constant.

CONCLUSIONS.

1. In addition to the paratyphoid bacilli already named there exists a group which occurs in a variety of animals and which culturally is the same as *Bacillus schottmülleri*. As a rule this group can be separated from the latter by the type of clumps formed when bouillon cultures are used as antigens, while other antigens and complement fixation tests have failed to differentiate it. Agglutination absorption tests sharply separate the animal from the human paratyphoids.

2. No differences have been detected between organisms of this group derived from a number of animals and a common name for them is desirable, but for the present it seems better to call them calf-,

swine-, mouse-, etc., typhus, according to the animal from which they were isolated.

3. Evidence exists in the literature that these organisms have been associated with food infections in man, particularly with what have been called paratyphoid B infections, but this function, as well as the part they play in animal diseases, is a subject for further study.

4. Well defined groups of paratyphoid such as *Bacillus cholerœ suis*, the Voldagsen bacillus, *Bacillus abortus equi*, and *Bacillus enteritidis* are found in animals in addition to the organisms considered in this paper, and every attempt should be made to range newly isolated organisms in one or the other of these well recognized groups.

5. One of the objects in continuing this work was to find a method of differentiating these animal from the human paratyphoids less complicated than agglutination absorption. This object was not realized; the two groups are very similar and agglutination absorption seems to be the only means of classifying them.

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BACILLI OF THE HOG-CHOLERA GROUP (BACILLUS CHOLERÆ SUIS) IN MAN.

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Hirschfeld (1) has described an epidemic of clinical paratyphoid fever which occurred in Serbia or Greece from which he obtained organisms culturally paratyphoid B or *Bacillus schottmülleri*, but which were not agglutinated by the sera of animals immune to the latter bacillus. These organisms were isolated eighteen times, twice being obtained after death. Sera obtained from patients in this epidemic agglutinated the organisms isolated in some cases in dilutions as high as 1:800. MacAdam (2) obtained similar inagglutinable paratyphoid bacilli in Mesopotamia from the blood stream of patients who clinically showed respiratory rather than enteric symptoms. Mackie and Bowen (3) have described cultures of the same group, as shown by Schütze's (4) work. The latter compared twelve cultures obtained from febrile cases occurring in the Balkan region, the organism isolated by the above writers being included. All the cultures were agglutinated by and absorbed the agglutinin from the serum of an animal immune to one of Hirschfeld's strains. Hirschfeld called his organisms paratyphoid C and Schütze called them Hirschfeld's bacillus.

It seemed possible that by comparing these organisms with the paratyphoids found in animals they might be classed in one of the known groups. Upon request Dr. Hirschfeld kindly sent me two cultures of these organisms and some immune serum. The cultures were labeled Para C and it may be that they were duplicates but I have called them Paratyphoid C I and II and have used both of them in the tests made. No differences have been detected between the two strains.

Agglutination tests were made with the serum received and the results given in Table I were obtained.

The serum agglutinates two strains of the hog-cholera bacillus in as high dilution as it does the organism isolated by Hirschfeld, while *Bacillus schottmülleri*, *Bacillus enteritidis*, and swine-typhus bacilli are agglutinated in only the lowest dilutions.

Anti-hog-cholera bacillus serum was absorl   he cultures received and the results of agglutination tests with the absorbed as well as the unabsorbed serum are given in Table II.

The paratyphoid bacilli under consideration are agglutinated to the titer limit by anti-hog-cholera bacillus serum ,   al rb from this serum not only the agglutinins for themselves bui als ʰose for the hog-cholera bacilli.

TABLE I.

Agglutination with Hirschfeld Paratyphoid C Serum.

Culture tested.	Limit of agglutination.
B. schottmülleri 232..	100
" " 242..	100
Swine *enteritidis* I..	50
B enteritidis (Mt. Sinai)....................................	50
Swine-typhus I..	100
" V	100
Hog-cholera XII	12,800
" XIV	12,800
Paratyphoid C I..	12,800
" " II	12,800

TABLE II.

Tests with Anti-Hog-Cholera Bacillus Serum.

Culture tested.	Limit of agglutination using serum.		
	Unabsorbed.	Absorbed with Paratyphoid C I.	Absorbed with Paratyphoid C II.
Paratyphoid C I..........................	12,800	200—	200—
" " II..........................	12,800	200—	200—
Hog-cholera bacillus XII...................	12,800	200	200
" " XVI.................	12,800	200	400

Rabbits were immunized, one to each of the two strains, by the injection of unheated bouillon cultures and their sera gave the results in Table III.

Four different hog-cholera bacillus cultures were agglutinated to the titer limit. Three of the hog-cholera bacillus cultures were used for absorption tests, one for one serum and two for the other, and they

took out the agglutinins for the Paratyphoid C bacilli, as well as for the other hog-cholera bacilli.

Serologically these organisms are typical hog-cholera bacilli but culturally they differ in that they form acid and gas in dulcite and arabinose and form hydrogen sulfide, whereas the hog-cholera bacillus, as has been pointed out by Jordan (5) and Krumwiede, Kohn, and Valentine (6), do not act on these carbohydrates or form hydrogen sulfide. Culturally, then, they are the same as *Bacillus schottmülleri*.

Two rabbits given subcutaneous injections of 0.1 cc. of 24 hour bouillon cultures of these organisms showed a slight rise in temperature

TABLE III.

Saturation of Paratyphoid C Sera with Hog-Cholera Bacilli.

Culture tested.	Limit of agglutination with serum of Rabbit A, immune to Paratyphoid C I.		Limit of agglutination with serum of Rabbit B, immune to Paratyphoid C II.		
	Unabsorbed.	Absorbed with Hog-cholera bacillus XVI.	Unabsorbed.	Absorbed with Hog-cholera bacillus X.	Absorbed with Hog-cholera bacillus XII.
Hog-cholera X, Pig 30	12,800	200	12,800	400	200−
" XI	12,800	200−	12,800	400	200−
" XII	12,800	200−	12,800	800	800
" XVI	12,800	200−	12,800	400	400
Paratyphoid C I	12,800	200−	12,800	400	800
" " II	12,800	200−	12,800	400	800

and local lesions. Neither was very sick and recovery was prompt. A typical hog-cholera bacillus should kill rabbits in from 6 to 10 days, so that these cultures resemble the animal typhus group rather than the hog-cholera bacilli in their virulence for rabbits.

20 days after the subcutaneous injection these rabbits were given an intravenous injection of 0.01 cc. of 24 hour bouillon cultures of the same organisms. This produced no effect and 25 days later they were given a subcutaneous injection of 0.00001 cc. of a 24 hour bouillon culture of a virulent hog-cholera bacillus. This organism in a dilution 100 times the one used here will kill normal rabbits in from 6 to 10 days, but in these injected rabbits it produced no rise in temperature, loss in weight, or local lesion. The rabbits were

killed 6 weeks after the final injection and aside from a slight enlargement of the spleen appeared normal. Cultures from the spleen showed hog-cholera bacilli from one rabbit but not from the other.

The interpretation of this experiment is not clear. As has been pointed out in the preceding paper (7), various animal typhus cultures will immunize rabbits to this virulent hog-cholera bacillus, but no tests have been made with the amount used here. The fact that these rabbits withstood such a large injection without showing any evident disturbance seems to indicate that they had a specific immunity.

A pig weighing 30 pounds was fed 100 cc. of one of the cultures received from Dr. Hirschfeld mixed with its food. For the next 4 days its temperature was increased and it ate very little. Its feces were diarrheal in character but the organism fed was not obtained on Endo plates made from the feces the 5th day after the feeding. On this day its temperature approached normal, it was eating well and appeared to be much livelier than on the days following the feeding. On the 5th day after the feeding virulent hog-cholera virus was injected intramuscularly, as it has been found in similar experiments that hog-cholera bacilli introduced into the digestive tract by feeding will act as secondary invaders when the pig is infected with virus. The pig died 8 days after the injection of the virus and was autopsied soon after death. It showed lesions characteristic of hog-cholera with the addition of a grayish, membranous necrosis of the mucosa of the large intestine. Plate cultures were made from the spleen, two mesenteric lymph nodes, the exudate in the colon, the mucous membrane of the colon after removing the exudate, the kidney, and the liver. In all, thirty-five subcultures from these plates were studied. They resembled the organism fed, with the following exceptions. Of six cultures from the mucosa of the colon, one failed to form hydrogen sulfide or ferment dulcite, but acted on arabinose. The other cultures were the same as the culture fed. Of two cultures from the kidney, one was the same as the organism fed, while the other differed in that it failed to act on dulcite. It did, however, produce hydrogen sulfide and on the second test fermented dulcite promptly. Only two cultures from the liver were examined and neither acted on dulcite, while one did and the other did not ferment

arabinose. Both formed hydrogen sulfide and on the second test both fermented dulcite and arabinose. The culture from the colon is, then, the only one that has shown any permanent change, and this culture 2 months after its isolation still failed to form hydrogen sulfide or ferment dulcite. It has, then, approached the hog-cholera bacillus in its cultural characters. The organisms from the liver and kidney also approached the hog-cholera bacillus in cultural character, but they soon regained the properties lost. The question as to whether the cultures recovered are the ones fed cannot positively be decided. Seven other pigs of the same litter have been infected with hog-cholera virus and bacteriological examination has failed to show the hog-cholera bacillus, though from some of them swine-typhus bacilli have been isolated. All the cultures from this pig resembled the hog-cholera bacillus serologically so the probabilities are that they were the descendants of organisms fed.

One similar test has been made with a swine-typhus culture and in this one case the feeding failed to cause a rise in temperature. Cultures made at autopsy, the animal having been infected with hog-cholera virus, failed to show swine-typhus bacilli.

When hog-cholera bacilli are fed, the pig reacts as did the animal fed the Paratyphoid C culture. There is an increased temperature beginning the day after the feeding and lasting for from 3 to 4 days. If at the end of this time hog-cholera virus is injected into the pig, hog-cholera bacilli will be found in the organs at autopsy.

DISCUSSION.

While these organisms isolated by Hirschfeld (1) are not typical hog-cholera bacilli in that they ferment dulcite and arabinose, produce hydrogen sulfide, and are not virulent for rabbits, their serum reactions are so characteristic that they should be placed in the hog-cholera bacillus group. These serum reactions are very fundamental, much more so than are the fermentations of the rarer carbohydrates.

There are several possible explanations which might account for these differences. One is that in the region from which these organisms were obtained atypical strains of hog-cholera bacilli exist in swine. Another is that the organisms in swine may be typical but

after passing to man they have become modified.   A third possibility is that these organisms did not come from swine.   They do not, however, correspond to any of the animal paratyphoids that have been described.   What appear to be culturally typical hog-cholera bacilli do exist in nearby regions, as is shown by the observations of Trawinski (8).   He isolated forty-two cultures from swine imported into Germany from Poland.   All the cultures failed to ferment dulcite and arabinose, while three cultures of so called *Bacillus suipestifer* obtained from Kral's collection acted on these carbohydrates.   One so called *suipestifer* strain obtained from Budapest acted the same as the cultures he isolated and was agglutinated to the titer limit by serum of an animal immune to one of his strains.   The cultures from Kral were not agglutinated to the titer limit by this serum.   He does not record the virulence of the cultures for rabbits or the production of hydrogen sulfide, but notes that all of his forty-two cultures formed acid but no gas in sorbite, whereas the control cultures of *Bacillus suipestifer* formed gas.

It is an interesting fact that the hog-cholera bacillus, which at one time was so commonly present in swine infected with hog-cholera that it was regarded as the cause of the disease, has not been found more frequently in man.   In the older literature of food poisonings some of the organisms isolated were virulent when injected subcutaneously into rabbits and in some cases necroses were found in the livers of these animals.   These facts indicate that the hog-cholera bacillus may have been the organism that was being studied, but the evidence is not conclusive.

Reed and Carroll (9) made a comparative study of *Bacillus icteroides* (Sanarelli) and the hog-cholera bacillus and concluded that they were the same.   In cultural characters, virulence, and the disease produced in animals the two cultures were identical.   They made only a few agglutination tests and the results are not very clear-cut but they indicate a relation between the two organisms.   We are fortunate in having in our collection a culture of *Bacillus icteroides* that was received directly from Sanarelli and it is agglutinated to the titer limit in anti-hog-cholera bacillus serum and absorbs the agglutinins from this serum.   In addition it fails to ferment dulcite and arabinose and does not produce hydrogen sulfide, thus resembling the hog-

cholera bacillus, while it differs from the latter organism in that it is not virulent for rabbits.

There is another culture in the collection labeled paratyphoid B Longcope, which culturally and serologically is a hog-cholera bacillus but which is not virulent for rabbits. The chances are that this culture came from a case of paratyphoid reported by Longcope (10) in 1902, but we cannot be sure of this fact. The probabilities are that it is at least of human origin.

As far as I know these are the only cultures from man that correspond closely to the hog-cholera bacillus so that an outbreak in the Balkan region with which organisms of the hog-cholera bacillus group are associated is of great interest. There must be many opportunities for hog-cholera bacilli to infect man, but they either rarely find conditions such that they can grow in the human body or, what is less likely, they do grow and quickly lose their distinguishing characters.

CONCLUSIONS.

1. The organisms isolated by Hirschfeld from febrile cases resembling paratyphoid fever and named Paratyphoid C can be placed in the hog-cholera bacillus group by their agglutination absorption properties though they are not typical culturally.

2. When fed to a pig a febrile disease resulted from which the animal recovered. After injection of hog-cholera virus the organisms fed were found generally distributed and some of them had lost cultural characters so that they are brought into the class of typical hog-cholera bacilli except for their low virulence for rabbits.

3. While hog-cholera bacilli have many opportunities to infect man they either are not able to grow in the human body or, what is less likely, they do grow and lose the characters that distinguish them.

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BLACKHEAD IN CHICKENS AND ITS EXPERIMENTAL PRODUCTION BY FEEDING EMBRYONATED EGGS OF HETERAKIS PAPILLOSA.

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The occurrence of blackhead or typhlitis in chickens has been noted by several observers. To the meager literature reported by Smith[1] in 1915 the following may be added.

In 1907 Theobald[2] reported the occurrence of the disease in England. In 1908 Milks[3] observed it in four widely separated localities in Louisiana. It was not seen in birds more than 6 weeks old. The course of the disease was rapid and the mortality 30 to 50 per cent. Higgins[4] found in one chicken ulceration of one cecum which he describes as typical of enterohepatitis or blackhead in turkeys. Tyzzer[5] observed the disease in two chickens. In a chick 4 weeks old there was found a slight invasion of the cecum and extensive involvement of the liver. A slight involvement of one cecum was later found in a hen 2 years old.

During the past 3 years a few cases of disease in chickens associated with *Amœba meleagridis* were brought to the laboratory. In the brief notes given below Nos. 18 and 19 were from one flock, and Nos. 91 and 92 from another. These flocks were over 50 miles apart.

Chicken 18, White Leghorn, brought alive Apr. 18, 1917. Said to have been hatched in Feb. At the autopsy there was found an intussusception of the small intestine of which about 7 cm. were involved. This portion was in a hemor-

[1] Smith, T., *J. Med. Research*, 1915, xxxiii, 243.

[2] Theobald, F. V., *2nd Nat. Poultry Conference, Official Rep.*, Reading, 1907, 181.

[3] Milks, H. J., *Louisiana Agric. Exp. Station, Bull. 108*, 1908, 1.

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[5] Tyzzer, E. E., *J. Med. Research*, 1919, xl, 1.

rhagic condition. Each cecum contained a consistent fecal mould coated with a thin but tough, partly white, partly grayish exudate. The walls of the ceca were thickened, opaque. The liver contained quite small, scattering, indistinctly outlined, yellowish foci. Sections of the ceca showed complet ' of 'he mucosa which was replaced by a dense layer of fibroblastic tissue. L w ' layer in the muscular coat and extending to the serous covering, there we umerous vacuoles of various sizes in the tissue which contained each from one to a dozen or more roundish bodies about 6μ in diameter. Each contained a n...leus with a minute deeply stained caryosome in state of binary division in some i the organisms. In the liver sections the foci seen at autopsy were about one-third the diameter of a lobule. They consisted of necrotic liver cells, parasites like those of the cecal walls, chiefly within giant cells, and many lymphoid elements.

Chicken 19, White Leghorn, received at the same time and from the same flock. The ceca were affected as in No. 18, but the liver was free from foci. Sections of the ceca showed the same kind of changes as in No. 18, but portions of the mucosa were still present. Parasites were rare and seen only in some of the many vacuoles scattered through the superficial fibroid substitute for the destroyed parts of the mucosa. These vacuoles probably lodged parasites now destroyed.

Chicken 91, Rhode Island Red, had been killed before reaching the laboratory. Female, weighing 500 gm. Both ceca contain consistent cores of fecal material which are not adherent to the wall. The underlying mucosa is smooth and free from necrotic areas. The liver is enlarged and covered with opaque whitish foci 6 to 7 mm. in diameter. Some contain minute yellowish specks in the whitish ground. In teased preparations from these foci numerous homogeneous, roundish bodies, about 12μ in diameter, are seen. These are not distinguishable from *Amœba meleagridis* found in diseased turkeys. In sections of the ceca, the thickening of the wall is due to a marked increase of plasma-like cells in the intertubular tissue. Small groups of *Amœba meleagridis* are scattered through the mucosa and one large group is located in the muscular portion of the intestinal wall. Coccidia are scattered through the core. Sections of the liver contained large numbers of *Amœba meleagridis*. This organ was involved as extensively and intensively as in fatal cases of blackhead in turkeys, while the lesions of the ceca were slight.

Chicken 92, Rhode Island Red, was received dead from the same flock. In this bird coccidiosis predominated and the only traces of blackhead found were two microscopic foci in the liver tissue crowded with *Amœba meleagridis*.

From the preceding quotations and studies it is obvious that fatal blackhead is relatively rare in chickens. The data indicate that it may be a quite common disease which passes unnoticed because partial or total recovery is the rule. The injury to the mucous membrane as shown in Cases 18 and 19 may prevent the recovered bird from reaching normal full development. The lesions and the asso-

ciated parasite appear to be identical with those observed in turkeys. The disease shows, however, greater variations from case to case. In some the liver is the chief seat and the ceca almost intact. In others the reverse is true.

The Experimental Production of Blackhead.

Following the successful attempts to produce blackhead in turkeys by feeding embryonated eggs of *Heterakis papillosa*,[6] similar experiments were made on chickens.

Experiment 1.—The chickens were hatched in an incubator. The eggs before incubation had been washed to remove any adhering dirt, placed in 0.5 per cent bichloride of mercury for 30 seconds, washed again, and dried. During the experiment the chicks were k pt in brooders within isolation units, protected against vermin and infectious material likely to reach them if kept in the open. The only source of infection to which they were exposed was the grain and sour milk fed regularly with cooked food.

The worms were obtained from the ceca of two adult hens penned with old turkeys in an outdoor enclosure since the fall of 1919. To liberate the ova, the females were cut up in a Petri dish containing a shallow layer of physiological salt solution. In this they were incubated at room temperature. The feeding was done Mar. 1, when the cultures were 14 days and the chicks 32 days old. Four White Leghorns, four Plymouth Rocks, and four Rhode Island Reds were fed the ova mixed with the food in cages. After the feeding their feet were thoroughly washed and they were placed in a brooder in an isolation unit. From the same hatch nine chickens were held as controls in another unit. The chickens were killed and autopsied at certain intervals after the feeding. In case the ceca were diseased, one was opened and examined as to contents and condition of the mucosa and the other simply incised and then placed in Zenker's fluid for future study.

Chickens killed respectively 1, 2, 3, and 7 days after feeding were found normal, except for weakness of the legs, due presumably to confinement. Sections of one cecum and of liver tissue showed normal conditions. The contents of the ceca consisted of bacteria, a few food remnants, and some *Heterakis* larvæ. The walls were free from infiltrations and contained only the usual number of lymphoid cell groups. In the livers a few small compact groups of cells resembling lymphocytes were present.

In No. 279, killed 10 days after feeding, both ceca were distended and contained a firm reddish core replacing the usual soft contents. The walls of the nonvillous portion were quite uniformly thickened to about 1 mm. Larval worms

⁶ Graybill, H. W., and Smith, T., *J. Exp. Med.*, 1920, xxxi, 647.

(*Heterakis papillosa*) were found in the lumen.   The liver was of normal appearance.   Transections of one cecum fixed without disturbing the core showed a number of changes.   The core, replacing the normal fecal mass, consisted almost wholly of red blood cells, only a few of which had retained the hemoglobin.   There were also some necrotic tissue cells in the mass.   Throughout the clot were small colonies of bacteria and a few larval forms of *Heterakis*.   The mucosa was greatly altered.   In several places it was completely destroyed.   Elsewhere, the tubules were scarce.   They were distorted and oblique to the surface.   The mucosa and submucosa contained large groups of lymphocytes and strands of the same were lying in the muscular tissue.   Parasites resembling *Amœba meleagridis* were abundant in the mucosa and submucosa.   Many were within giant cells.   The muscular coats were free.   Sections of a small nematode were found in the intertubular tissue, measuring about 0.05 mm. in diameter.   In the liver there were fairly numerous dense collections of lymphocytes up to 0.15 mm. diameter.   Parasites could not be detected in these foci.

No. 280, killed 11 days after feeding, presented the same lesions.   Neither this nor the preceding bird had shown evidences of disease.   The core in the cecum was like that of No. 279.   The wall presented certain differences, however.   The epithelium and the tubules were normal and intact.   The submucosa was markedly edematous and the lymphoid tissue somewhat increased.   The blackhead parasite had permeated quite generally and distended the intertubular tissue of the mucosa and had penetrated into the submucosa in large numbers.   Worms were seen in the mucosa and the lumen.   The cell foci as described under No. 279 were present in the liver of this case.

Chicken 281 was killed 14 days after feeding ova.   It had not shown signs of illness.   The contents of one cecum were normal and contained larval worms.   The wall was possibly slightly thickened and the mucosa sprinkled with minute hemorrhages.   Sections of the other cecum showed conditions differing both from the preceding and the following case.   The contents were normal.   A small portion of the wall was normal, the rest thickened.   The tubules and surface epithelium were intact.   One larval worm was found partly embedded in a tubule.   The increased thickness of the wall was due chiefly to a great increase in lymphoid cell groups in the submucosa.   In the mucosa there was a slight diffuse infiltration of plasma cells.   The muscular coat was not involved.   *Amœba meleagridis* occurred in groups of two to six or more individuals in tissue spaces.   The parasites were relatively scarce as compared with No. 279.   The liver contained minute collections of lymphoid cells, from one to two in a field of the 16 mm. objective.

Chicken 282 was killed 15 days after feeding.   It had not appeared quite normal.   Both ceca were distended and firm to the touch, owing to the presence of hemorrhagic cores replacing normal feces.   The walls were 2 to 4 mm. thick.   Larval worms were present.   The core was made up of a mass of red corpuscles embedded in a homogeneous feebly stained matrix.   In one spot it was incorporated with the wall and here the mucosa was destroyed.   The rest of the mucosa was covered with epithelium but the tubules were partly destroyed, partly dis-

torted. The intertubular tissue was infiltrated with lymphoid cells and amebæ. The submucosa was markedly edematous. At irregular intervals it contained large dense collections of cells enclosing amebæ. In a few places the muscular coat was infiltrated with lymphocytes.

Chicken 285, killed 18 days after feeding, had not shown symptoms of illness. It was normal as to ceca. There were a few whitish foci 1 to 2 mm. in diameter in the liver. Transverse sections of fixed and hardened tissue from four different levels of one cecum showed the same changes. There were a cellular infiltration and increase of lymphoid tissue in the mucosa and a few tubules were markedly distended. Just below the muscularis there was a dense zone or layer of cells, probably lymphocytes, and roundish masses of the same, suggesting newly formed follicles. There was no core or mass of exudate in the lumen and there were no parasites in the tissues. In the liver sections, besides the minute collections of lymphoid cells, there are a few larger groups of the same type of cells enclosing necrotic liver tissue. *Heterakis* larvæ were in the ceca.

Chicken 286, killed 23 days after feeding, had not shown symptoms. The contents of the ceca were normal and larval worms were present. There was evidence of increased numbers of cells, resembling plasma cells in the mucosa. Lymphoid follicles were more numerous than normally in the submucosa, but the diffuse infiltration of lymphocytes was lacking.

Chicken 287 was killed after 28 days. It had not shown symptoms. One cecum contained some *Heterakis* larvæ, but contents were normal. Sections of the other cecum showed slight irregularity and occasional loss of tubules, their places being filled with cells of lymphocyte type. In the intertubular tissue there was an increase of plasma-like cells. The submucosa contained more than the normal number of lymph follicles. There was one group of eighteen follicles. In the liver certain whitish foci, 1 mm. in diameter, seen at the autopsy, were found to be made up entirely of cells of lymphocyte type. A central mass about 0.5 mm. diameter was enclosed in a ring of follicles. Many small foci of similar cells, 0.1 to 0.15 mm. diameter, frequently filling out vessels were also present.

Chicken 288 was killed after 29 days. *Heterakis* larvæ in ceca. Although the animal had shown no distinct signs of disease and the organs appeared normal with the exception of a few whitish areas on the liver 1 to 2 mm. in diameter, the walls of the ceca were not normal. The mucosa had the usual number of tubules with the exception of about one-tenth of the circumference, in which the tubules were replaced with lymphoid cells. There was a general increase of cells of plasma type between the tubules and the number of follicles in the submucosa was increased. In a section of the liver there were two foci, one consisting of a plug of cells in a vessel, the other a group of cells simulating four lymph follicles surrounding a small vessel also plugged with lymphoid cells.

Putting the data of this experiment together, we observe that as a result of hemorrhages the contents of the ceca appeared as firm cores

on the 10th to the 15th day inclusive, but not before the 10th, nor after the 15th day. Protozoan parasites were in the walls of the ceca within the same period. A larger number of cases examined at shorter intervals will define these limits more accurately.

Of the controls, which had remained well with the exception of some lameness due to confinement, two were killed 17 days after the other lot had been fed, and one 30 days after. None showed signs of lesions and worms were not found.

Experiment 2.—In this experiment, which is similar to Experiment 1, certain controls were introduced. The chickens were obtained from a poultry farm just after they had been hatched and placed in a brooder in an isolation unit. They were fed as were those in Experiment 1. The worms used were obtained from the same source as those used in Experiment 1. The cultures were prepared in the same way as heretofore. Three groups were included, each consisting of four White Leghorn chickens. Group I was fed with cultures made by cutting up adult female *Heterakis* to permit eggs to escape. Group II received cultures prepared by cutting up only males. Group III was fed with sediment from the washed contents of the ceca after the worms had been removed and the contents run through a wire screen of No. 300 mesh which did not permit the few free ova to pass through. At the time of feeding, the cultures were 20 days and the chickens 18 days old. All of the chickens in Group I were diseased, as the following notes show.

On the 9th day after feeding No. 290 was not quite normal. Its head was drawn back and its wings drooped slightly. It was chloroformed next day. The body was in good condition and only the ceca were affected. They were distended to about 1 cm. in diameter, firm to the touch, and hemorrhagic at the distal end. One, cut open, contained a spongy pink and reddish core slightly adherent to the hemorrhagic mucosa. The liver showed some whitish specks. Transverse sections from the other cecum showed the presence of a core made up of a meshwork of a homogeneous substance containing some red corpuscles. It was attached to the greater part of the wall and the mucosa was here destroyed. The remaining mucosa was low, with continuous epithelium and distorted tubules. The submucosa was broadened by infiltration with large numbers of *Amœba meleagridis* and lymphoid cells. The muscular layers were also infiltrated with cells, even into the mesentery. Larvæ were present in the mucosa and lumen. In the liver sections only minute groups of lymphocytes were present.

Chicken 295 was not quite normal on the 11th day, when it was chloroformed. The gross appearance of the ceca and the condition of the wall and core of one cecum as shown in sections were so like those of No. 290 that a detailed statement is omitted. In the liver a necrotic focus with a few *Amœba meleagridis* in it deserves mention.

Chicken 296 was killed on the 12th day. It had not shown any distinct signs of illness. Both ceca were distended and firm. One was opened and a long, cylindrical, non-adherent core, gray and brown in color, removed. The wall was about 2 mm. thick. In stained sections, the core was found composed chiefly of exudate cells (lymphocytes). It was attached at one spot to the wall and here the mucosa had been destroyed. Elsewhere the low mucosa was covered with epithelium. Normal tubules were scarce. The rest were much dilated and filled with cell debris. The mucosa, submucosa, and muscular layers were indistinguishable, owing to a general invasion of *Amœba meleagridis* and a general infiltration of cellular elements, lymphocytes, and plasma cells. There were also in the muscular coat masses of cells simulating lymph follicles. In the liver there were the usual minute collections of lymphocytes and some necrotic foci, but parasites were not seen in them.

The 4th and last chicken of Group I, No. 298, was killed 13 days after feeding. It had been clinically normal. Both the macroscopic and microscopic pictures were so like those of No. 296 that no detailed description is given. The invasion of *Amœba meleagridis* was slight below the submucosa, but the infiltration of the musculature with large masses of lymphocytes, even into the mesentery, was striking.

· In three of the foregoing cases larval worms were found in the contents of one cecum when examined fresh. In the fourth they were demonstrated in transections of the core of the other cecum.

The four chickens of Group II remained normal until they were killed on the 10th, 11th, 13th, and 13th day, respectively. No lesions were found and worms could not be detected. The same was true for the chickens of Group III, which were killed on the 10th, 11th, 12th, and 13th day, respectively.

DISCUSSION.

The foregoing experiments demonstrate that a disease of the ceca very closely resembling that of the enterohepatitis or blackhead of turkeys can be produced in chickens still in the brooder by feeding an overdose of embryonated eggs of *Heterakis papillosa*. The lesions appeared on or about the 9th day after feeding. Chickens killed after 15 days showed, in spite of an intact mucosa, signs of a past inflammation in the presence of large numbers of cells of the lymphocyte type in mucosa and submucosa and in the increased number of roundish accumulations of similar cells resembling lymph follicles.

The active disease manifests itself in a pouring out of blood into the lumina of the ceca, which coagulates into a firm spongy core. Later the red corpuscles disappear and lymphocytes emigrate from the

injured mucosa to form the outer zone of the core.   This is attached in part to the wall and here the mucosa is necrotic.   The walls of the ceca are thickened up to 4 mm. in diameter.   The thickening is due to edema, infiltration of lymphocytes and of *Amœba meleagridis* in large numbers.   The mucosa is more or less injured in addition to the necrosis where the core is adherent.   The tubules are distorted, dilated, and in part missing.   Some are filled with cell debris.   In all cases there is a diffuse infiltration of plasma-like cells between the tubules.   Occasional hemorrhages into the substance of the mucosa are present.

The liver, besides containing in all cases microscopic focal collections of lymphocytes, shows rather infrequently barely visible yellowish specks which consist either of necrotic foci in which giant cells and cells of endothelial type are replacing the liver cells or else of collections of lymphocytes in the form of roundish follicles or within vessels.   *Amœba meleagridis* was seen in one case in a necrotic focus. On the whole, the changes in the liver so formidable in turkeys are insignificant.

, The protozoan parasites presented the same morphological characters shown in the turkey's tissues.   They occupied the tissue spaces in mucosa and submucosa and more rarely in the muscular coat.   As a rule, the bodies of the parasite appeared as if in a state of disintegration except in No. 281, in which they had a homogeneous cytoplasm. In some cases most of them were within phagocytic cells.   In one case (No. 290) the protozoa set free from teased portions of the mucosa showed, without the use of a warm stage, finger-like pseudopodia in continual change.

The genesis of the clot in the ceca is not clear.   The cases studied were not timed so as to encounter it in its formation.   This gap must be filled before any basis for a discussion of the nature and significance of the cecal lesions can be found.

In general the larval stages of *Heterakis* were present but in small numbers.   In four cases individuals were detected in the mucosa itself.

Flagellates so common in the tubules of the turkey were entirely absent in the two series of chickens.

The injurious effects of feeding embryonated eggs of *Heterakis papillosa* to young chickens are no less definite than those observed after feeding them to turkeys. There are, however, certain distinctions to be drawn between the disease in turkeys and in chickens. The turkeys were found much more susceptible since the feeding produced uniformly a severe disease probably fatal in all birds if those that were chloroformed had been allowed to live longer. The effect on the condition of the young chickens was slight and they would probably have all survived.

The lesions due to the feeding differ materially as regards the liver. In turkeys this organ is almost uniformly invaded by *Amœba meleagridis* and the resulting foci of multiplication lead to a destruction of a variable amount of liver tissue, often over 50 per cent. In chickens the invasion is so slight that when it does take place the resulting lesions are scarcely more than microscopic in size. The liver is not wholly immune, however, as spontaneous cases now and then prove.

As to the ceca, the lesions induced in chickens appear formidable enough, but they probably undergo speedy resolution and the destroyed mucosa is covered with epithelium in due time. The after effects of the partial destruction of the mucosa may be more serious and tend to interfere with the normal growth of the chicken. This destruction may be permanent and lead to a replacement of the mucosa by scar tissue, as shown in the two spontaneous cases described above. In general it may be assumed that injury to the ceca due to *Heterakis* and *Amœba meleagridis* is not uncommon and its causal relation to other pathological conditions of poultry may be far reaching. Not until this worm has been largely suppressed can the extent of the injury due to it be inferred in retrospect. The degree of injury inflicted appears to be largely a question of dosage. The more ova ingested the more widespread and intensive the lesions, whereas the ingestion of a few does not appear to be dangerous even to turkeys.

Indications of a tendency of the nematodes to encyst in the walls of the ceca, as observed by Letulle and Marotel[7] in a pheasant, were wholly absent in the cases examined.

The presence of *Amœba meleagridis* in chickens still in the brooder after they have been fed ova either free or still within fragments of

[7] Letulle, M., and Marotel. *Arch. parasitol.*, 1908, xii, 361.

worms incubated in physiological salt solution for 15 to 20 days at room temperature points to the presence of *Amœba meleagridis* in the cultures. This cannot be confirmed or disproved until some method other than feeding *Heterakis* eggs is found which will induce conditions favorable to the invasion of *Amœba meleagridis* into the walls of the ceca. So far it has been impossible to start the disease by feeding incubated feces or cultures from which the worms and ova had been removed. It should be stated that protozoa resembling amebæ and flagellates have been found in the cultures of ova fed and a study of these is now under way. The relation of *Heterakis papillosa* to typhlitis in turkeys and chickens and the slightly varying morphology of the invading protozoa present for consideration the possibility that the latter may not necessarily belong to one species.

CONCLUSIONS.

Feeding embryonated eggs of *Heterakis papillosa* to brooder chickens led to a disease of both ceca, characterized by the presence of a core consisting of fecal matter, coagulated blood, and emigrated cells from the mucosa. The walls of the ceca were thickened as a result of cell invasion and multiplication, invasion and multiplication of *Amœba meleagridis* or allied parasites, and more rarely hemorrhage and edema. The respective parts played by *Heterakis papillosa* and the protozoa in starting the lesions and the source of the protozoa remain to be defined. The invasion of the liver by the protozoa was insignificant.

A STUDY OF BACILLUS PYOGENES.

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PLATES 14 TO 16.

INTRODUCTION.

Bacillus pyogenes is associated with various disease processes of swine and cattle and is not infrequently found in milk. Failure to recognize it as the *B. pyogenes* described in French, Dutch, and German literature may be due to certain difficulties in its cultivation, to its close resemblance to streptococci under certain conditions, and to the fact that it is usually found in mixed culture with organisms which may mask its presence.

In France, according to Lucet (1893), next to the streptococci *B. liquefaciens pyogenes* is one of the most frequently found organisms in suppurations of cattle. In Germany Künnemann (1903) found a similar organism, which he calls *B. pyogenes bovis*, in 90 per cent of suppurations of cattle. Grips (1898) found *B. pyogenes suis* commonly present in pleuritis and peritonitis of swine. Tuff (1906) found *B. pyogenes* in over 13 per cent of milk samples examined. Glage (1902–03) found the organism third in importance to streptococci and *B. tuberculosis* as a cause of mastitis in cows. Eggink is quoted by Ward (1917, *a*) as having found *B. pyogenes* of first importance in metritis of cattle. There are no statistics as to the prevalence or distribution of the organism in America. Ward (1917, *a*) found it frequently in swine and cattle.

Lucet (1893) studied 52 cases of suppuration, cold abscesses, traumatic abscesses, and cases of septicemia, all in cows. He says: It seems that there exist in the cow special pyogenic microbes, not yet described, which are a streptococcus, a staphylococcus, and three bacilli. He names these organisms *Streptococcus pyogenes bovis, Staphylococcus pyogenes bovis, B. pyogenes bovis, B. liquefaciens pyogenes bovis*, and *B. crassus pyogenes bovis*. His description of *B. liquefaciens pyogenes bovis* agrees with that of the *B. pyogenes* of Grips, Glage, Künnemann, and others. Lucet gives a photograph of the organism and describes it as non-motile, liquefying gelatin slowly, not growing on potato, growing as a sediment in veal bouillon without producing turbidity, non-virulent for guinea pigs, injected into rabbits intravenously producing subaponeurotic abscesses principally in the limbs where they sometimes acquire great size but do not discharge. His *B. pyogenes bovis*

resembled the above morphologically but did not liquefy gelatin and its pathogenicity for guinea pigs was variable. *B. crassus pyogenes bovis* was a larger motile bacillus. Grips (1898) described *B. pyogenes suis* and the lesions of the pleura and peritoneum from which it was isolated. Poels (1899) described a similar organism from polyarthritis of calves and called it the polyarthritis bacillus. Künnemann (1903) described *B. pyogenes bovis* from suppurations of cattle. Glage (1902–03) made a careful comparison of *B. pyogenes suis* (Grips) and *B. pyogenes bovis* (Künnemann) and concluded that they were identical. He proposed that the organism be called *B. pyogenes*. His contention that the organisms from swine and cattle are identical has not been seriously questioned. Careful bacteriological studies have been made by Koske (1906), Berger (1908), and Holth (1908). Ward (1917, *a* and *b*) has given valuable brief summaries in English, and Glage (1913) in German.

Source of Strains.

The strains of *Bacillus pyogenes* of bovine origin employed in this study were received from Dr. Theobald Smith who supplies the following data concerning their source.

Strain I. From pneumonic lungs of a calf. Killed when 33 days old.[1]

Strain II. From pneumonic lungs of a calf. Killed when about 5 weeks old.[1]

Strain III. From pneumonic lungs of a calf. Killed when 38 days old.[1]

Strain IV. From pus filling both horns and body of uterus of a cow. Uterus obtained after slaughter of cow.

Strain V. From a similar case as that of Strain IV. Uterus contained a foul smelling fluid.

Strain VI. From purulent contents of uterus in case of prolapse of vagina and external os. Uterus obtained when cow was slaughtered.

Strain VII. From pneumonic lungs of a calf. Killed when 31 days old.

Strain VIII. From the uterine contents and ovaries of a case of purulent metritis and of central necrosis and pus formation in both ovaries. Associated with *B. actinoides*.

Strain IX. From chocolate-colored, offensive fluid contained in uterus of a cow slaughtered. Other bacteria present.

Strain X. From kidney, liver, and lungs of the fetus of Cow 259. Several other species of bacteria present in small numbers.[2]

Strain XI. From the fourth stomach, liver, and lungs of the fetus of Cow 291.[2]

Strain XII. From the liver of the fetus of Cow 339. Pure culture of *Vibrio fetus* isolated from the lungs.

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[1] It is highly probable that in these cases *Bacillus pyogenes* was secondary to *Bacillus actinoides* (Smith, T., *J. Exp. Med.*, 1918, xxviii, 333).

[2] Smith, T., *J. Exp. Med.*, 1919, xxx, 325.

Strain S 1 was isolated by Dr. Carl TenBroeck from the pneumonic lung of a case of hog-cholera. Other organisms were also present in the lung.

Cultural Study.

The known morphological and cultural characteristics of *B. pyogenes* as described by Lucet (1893), Grips (1898), Poels (1899), Künnemann (1903), Glage (1902–03), Koske (1906), Berger (1908), and Holth (1908) have been summarized by Glage (1913), Buchanan and Murray (1916), and Ward (1917, b). They are briefly as follows:

The organism is a small slender rod 0.2 to 3.0 microns in length by 0.2 to 0.3 microns in thickness. It is quite pleomorphic being often coccoid, club-shaped, or slightly curved. It is non-motile and produces no spores. Some authors (Glage, Künnemann) have regarded it as Gram-negative and others as Gram-positive (Berger, Holth, Olt, Ward). Berger found it Gram-positive if subjected to sufficient exposure to the iodine solution. Capsules are not produced.

It is stated by most authors that the organism grows very poorly or not at all in standard bouillon or on standard agar, and that it requires hemoglobin, blood, or serum in the medium. Good growth occurs on blood or serum agar. Coagulated blood serum is slowly liquefied beginning in about 48 hours as small depressions underlying each colony. Because of this characteristic this medium has been a favorite one for isolating the organism. Milk is coagulated in about 48 hours and the curd is subsequently slowly dissolved or digested. In liquid serum or serum bouillon growth occurs in the form of a sediment. Growth does not occur at temperatures below 24°C. but in a specially prepared nutrient gelatin of high melting point Poels found liquefaction produced by the organism growing at 26°C. Growth occurs under aerobic and anaerobic conditions. Gas is not produced in carbohydrate media. According to Pütz (1904) acid is produced. Koske (1906) reports acid production in serum litmus whey. Berger (1908) obtained no growth in serum litmus whey and does not mention acid production in lactose or dextrose bouillon but notes that milk is coagulated and soured. Holth (1908) reports acid production from dextrose, fructose, galactose, maltose, lactose, and saccharose in a special meat extract (Cibil's) bouillon but obtained no acid or visible growth in the same medium containing xylose, rhamnose, arabinose, sorbose, mannitol, sorbitol, dulcitol, or glycerol. Indole, hydrogen sulfide, and nitrites are not produced. Methylene blue, litmus, and neutral red are not reduced. The bacillus is soon killed at 57°C. and is very sensitive to antiseptics.

Our experience with the organism agrees with the above as regards morphology, oxygen requirements, growth on coagulated serum, in milk, and in serum bouillon. In plain standard veal infusion bouillon made with Fairchild's peptone, however, we have obtained fairly

good growth, at least with strains which have been in cultivation a very short time. For a while the bouillon was not clouded by the culture but after cultivation for several months bouillon was distinctly clouded in 24 hours by most strains.

The production of hemolysis in blood agar by *Bacillus pyogenes* appears not to have been noted heretofore. In standard veal infusion agar plus 5 to 10 per cent of defibrinated horse blood there appear after incubation for 20 to 24 hours very small zones of hemolysis about very minute deep colonies. The colonies are often visible only under the low power of the microscope. In 48 hours the deep colonies are still quite small biconvex discs about 0.3 mm. in greater diameter but are easily seen macroscopically. The hemolyzed zones are clear, well defined, colorless, and of the beta type (Smith and Brown, 1914–15; Brown, 1919), about 1.5 to 2 mm. in diameter (Fig. 1). Isolated surface colonies do not appear so readily on the plate under aerobic conditions and the zone of hemolysis may be hardly visible. If the plate is sealed, however, individual surface colonies grow more readily and produce zones of hemolysis similar to those of deep colonies. If the blood agar plate is streaked so that many small surface colonies appear in the line of the streak, hemolysis appears beneath the streak. The individual surface colonies are very small convex colorless droplets much like those of *Bacillus influenzæ*.

We have sought to determine whether *Bacillus pyogenes* is hemoglobinophilic or whether it may be dependent upon other substances in blood for growth.

Freshly drawn horse blood was allowed to clot and a clear straw-colored serum was obtained as nearly free from hemoglobin as possible. This was used in serum agar plates.

Another portion of the same blood was defibrinated, and the corpuscles were washed repeatedly with sterile physiological salt solution. Some of the washed corpuscles were used in washed corpuscle agar plates.

Some of the washed corpuscles were laked with sterile distilled water and the corpuscle stroma removed by centrifugation. Care was taken to centrifuge the laked blood corpuscles until the supernatant hemoglobin solution no longer gave a clouding reaction with salt (Brown, 1919[3]). The hemoglobin solution so obtained was used in hemoglobin agar plates.

[3] Brown (1919), p. 67.

The corpuscle stroma obtained by centrifugation of the laked corpuscles was washed repeatedly in sterile distilled water and in salt solution until no visible trace of hemoglobin remained. The stroma suspension was used in stroma agar plates.

Each strain of *Bacillus pyogenes* was inoculated into the depths and streaked onto the surface of plates of the following media: (1) blood agar; (2) serum agar; (3) washed corpuscle agar; (4) hemoglobin agar; (5) stroma agar; (6) plain agar. Every precaution was taken to insure uniformity of conditions, such as use of the same lot of agar throughout, inoculation of one plate after another in the same manner and with the same amount of material. The plates were inoculated from fresh plain bouillon cultures. Observations were made as to the amount of growth, size and number of colonies, and morphology of the organisms within the colonies. In every case the best growth was obtained in blood agar. Next best was the growth in washed corpuscle agar and in serum agar. In the majority of cases better growth was obtained in stroma agar than in hemoglobin agar. Little or no growth occurred in plain agar. The results indicate that serum is of as much importance as corpuscles, and that hemoglobin is probably the least essential of the blood constituents for the growth of this organism. That hemoglobin does not satisfy the requirements of *Bacillus pyogenes* as it does those of *Bacillus influenzæ* is indicated by the following experiment. A blood agar plate was inoculated in the depths and also streaked with *Bacillus pyogenes*. At a point near the streak a large zone of laking was produced by depositing a bit of saponin on the surface of the medium. Both deep and surface colonies within the zone were no larger and no more numerous than elsewhere in the plate (Fig. 1). Under similar conditions colonies of *Bacillus influenzæ* grew more luxuriantly in and near the zone of laking than elsewhere in the plate. A similar result was obtained by producing zones of laking by directing a stream of carbon dioxide against the bottom of the plate until a spot was frozen. The blood used for such experiments must be fresh; otherwise there will be sufficient free hemoglobin in the serum to obliterate the difference in growth between that in the laked zone and elsewhere.

The fact that colonies of *Bacillus influenzæ* grow more luxuriantly in the vicinity of colonies of hemolytic streptococci and staphylococci has

been known for many years (Grassberger, 1897). According to Davis (1917) there is involved in this phenomenon not only hemoglobin but also a vitamine which is supplied by the foreign organism or may be supplied by fresh sterile vegetable or animal tissues. We have not found the growth of *Bacillus pyogenes* colonies to be augmented by proximity to colonies of other organisms.

That blood is not absolutely necessary for the growth of *Bacillus pyogenes* is shown by the fact that it grows very well on Dorset's egg medium. On this medium the colonies, especially those on the upper half of the slant, produce little pits like those produced on coagulated serum. The organism also grows fairly well on a medium consisting of three parts of white of egg plus one part of standard veal infusion bouillon, slanted and coagulated in the inspissator. On this medium the streak of growth also produces some depression as on coagulated serum. Even an old laboratory strain of *Bacillus influenzæ* which has become quite easy to cultivate grows very slightly on Dorset's medium and not at all on the egg white medium.

Staining and Morphology.

Bacillus pyogenes stains well with dilute carbolfuchsin or with Löffler's methylene blue but we have obtained the best results with the Gram stain. Under the influence of prolonged decolorization with alcohol the organism may not retain the violet stain so tenaciously as do staphylococci, but when stained on the same slide with *Bacillus influenzæ, Bacillus coli*, the meningococcus, or other recognized Gram-negative organisms there can be no doubt as to *Bacillus pyogenes* being clearly Gram-positive. The disagreement in the literature as to the Gram staining of the organism may be due to the many methods in use for applying and making up this stain and the poor keeping qualities of some of them. It is also pointed out by Olt (1908) that the dead organisms found in old exudates are Gram-negative whereas the living organisms are Gram-positive. We have used Stirling's aniline gentian violet, but 1 or 2 per cent of the dye by weight rather than 5 per cent has been found sufficient. Exposure of films to the violet stain for 10 seconds, Lugol's solution for 15 to 30 seconds, decolorization in alcohol until no more color appears to be given off,

and counterstaining for 10 seconds in aqueous safranine has always given good results.

The various media employed have afforded opportunity to observe the pleomorphism of *Bacillus pyogenes*. Four or five more or less distinct forms and their intermediate stages are recognizable.

1. Bacillary Form.—These are small, short, homogeneously stained Gram-positive bacilli occurring singly (Fig. 2).

2. Fusiform Form.—In this form there are one or two strongly Gram-positive central granules while the ends of the bacilli fade out and take the counterstain to some extent. These are often slightly curved, and superficially resemble *Bacillus acne* though smaller (Figs. 3 and 4).

3. Diphtheroid Form.—These are bacilli of irregular length and contour, some clubbed, containing deeply stained bands or granules irregularly placed (Fig. 6).

4. Streptococcoid Form.—These appear exactly as Gram-positive diplococci and streptococci in small clumps and short crooked chains. If, as may be supposed, these cocci are but the granules of the diphtheroid form which have assumed a very regular size, form, and arrangement, the matrix forming the remainder of the bacillus is invisible to the eye though the photographs reveal a faint matrix which probably retained a trace of the red counterstain (Figs. 7 and 8).

5. Filamentous and Branching Form.—The bacilli appear drawn out with irregular diameter. There are few definite deeply staining granules but in some filaments there may be a deep violet portion blending gradually with a faintly stained or Gram-negative portion. Definite buds and branches were occasionally found, especially in Strain IV (Figs. 5 and 6).

Cultures often show a mixture of the above forms. Some strains have more of a tendency to assume one form and others another, but all the strains studied have produced all the various forms at one time or another. The streptococcoid form may be so definite and so habitual with certain strains that the organism may be mistaken for a streptococcus. We have spent some weeks studying what was thought to be a very unusual minute hemolytic streptococcus only to discover that we were working with *Bacillus pyogenes*.

In observing the morphology of the organism on various media we hoped to discover the factors which determined the variations in form, but the hope was only partially realized.   In the lesions of animals the organism appears in the bacillary form with a tendency to be more granular in older lesions.   In bouillon and serum bouillon the bacillary form predominates though any or all of the other forms may also be present; the streptococcoid form is least likely to appear.   The latter form was encountered most commonly on washed corpuscle agar, stroma agar, and especially on hemoglobin agar.   On blood agar there was a mixture of bacillary, granular, and streptococcoid forms.   The growth on serum agar was bacillary.   Long filamentous and branching forms were obtained in serum bouillon and in the condensation fluid of coagulated blood serum.   Fusiform bacilli appeared most commonly in milk and in bouillon containing fermentable sugar. Our study has produced the impression that the filamentous and streptococcoid forms represent the two extremes of pleomorphism.   The strains which produced filamentous forms most readily, *e.g.* Strain IV, produced streptococcoid forms with difficulty, while other strains, *e.g.* Strain X, produced streptococcoid forms readily but filamentous forms rarely.   The forms most commonly encountered are the bacillary and fusiform.

Fermentation.

In fermented bouillon plus 10 per cent of sterile horse serum and 1 per cent of the test substance all the strains of *Bacillus pyogenes* produced acid from dextrose, saccharose, lactose, and xylose but not from raffinose, inulin, mannitol, and salicin.   Holth (1908) reports that galactose, fructose, and maltose are also fermented.   We differ from him in regard to xylose.   Our fermented bouillon containing the test substances was probably more favorable for the growth of the organism than was his meat extract bouillon.

Fermentation tubes were employed for the tests and the contents of both the open bulb and the closed arm were titrated for acid after incubation of the cultures for 7 days.   The results recorded in Table I show that the acidity of the bulb was much higher than that of the closed arm, little or no acid often being produced in the latter.   *Bacillus pyogenes* is completely agglutinated by normal horse serum in

dilution of 1:100. In dextrose serum bouillon the arm of the fermentation tube remains clear and most of the growth is in the form of a sediment in the neck of the tube. If, however, the fermentation tube be incubated in the horizontal position (Text-fig. 1) an abundance of

TABLE I.

Fermentation Reactions.

Strain No.	Xylose.		Dextrose.		Lactose.		Saccharose.		Raffinose.		Inulin.		Mannitol.		Salicin.	
	Bulb.	Arm.	Bulb.	Arm.	Bulb.	Arm.	Bulb.	Arm.	Bulb.	Arm.	Bulb.	Arm.	Bulb.	Arm.	Bulb.	Arm.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
I	3.85	2.15	3.35	1.4	4.25	2.45	1.95	0.8	0.9	0.4	1.1	0.75	1.5	0.55	1.1	0.9
II	4.1	1.9	3.55	2.1	5.35	2.1	3.25	1.9	0.75	0.35	0.9	0.5	0.65	0.75	0.7	0.7
III	3.8	2.05	3.35	1.8	5.5	3.05	2.3	1.45	1.0	0.65	0.6	0.6	1.0	0.65	0.7	0.6
IV	4.0	1.9	3.9	1.2	3.35	0.85	2.75	0.9	0.85	0.4	0.75	0.55	1.0	0.65	0.75	0.4
V	3.55	2.2	3.7	1.95	3.9	1.65	2.8	1.6	1.15	0.6	0.9	0.4	0.75	0.5	0.7	0.4
VI	4.8	2.55	3.4	2.1	2.0	1.6	2.65	1.05	0.95	0.55	1.05	0.7	0.8	0.4	0.65	0.65
VII	3.35	2.55	2.6	2.2	4.05	2.35	3.75	1.65	1.2	1.0	1.3	1.05	1.15	0.08	1.6	0.85
VIII	4.4	3.2	4.0	1.5	4.6	2.5	3.2	2.7	0.65	0.45	1.0	0.9	0.95	0.5	1.1	0.65
IX	2.9	1.2	2.65	1.45	1.85	1.1	2.85	1.85	0.5	0.4	0.8	0.4	0.4	0.5	0.75	0.45
X	3.1	0.7	3.4	0.85	3.9	1.4	3.25	1.35	0.85	0.5	0.85	0.35	0.6	1.2	0.65	0.5
XI	3.75	1.8	3.85	1.5	2.8	1.05	3.4	1.15	1.1	0.8	1.2	0.4	0.75	0.45	0.8	0.6
XII	3.35	2.35	3.0	1.35	1.8	1.2	3.5	1.3	1.0	0.65	0.9	0.55	0.75	0.7	0.9	0.65
S 1	3.75	1.35	4.15	1.4	4.1	1.25	1.8	0.6	1.45	1.0	1.2	0.6	0.6	0.4	1.0	0.9

The figures indicate per cent normal total titratable acid.
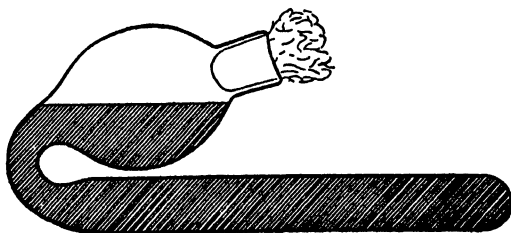
TEXT-FIG. 1. Fermentation tube placed in the horizontal position.

acid is formed in the arm as well as in the bulb. These facts suggested for the moment that failure to ferment in the arm might be due to the mechanical effect of agglutination. It was discovered, however, that in plain dextrose bouillon without serum the arm was well clouded by

growth but little or no acid was produced in it. The effect of difference in oxygen tension was next considered. The closed arm of a fermentation tube of sterile bouillon containing 1 per cent of a 1:1,000 aqueous solution of methylene blue reduced by autoclaving remains colorless for weeks if incubated in a vertical position. The arm of a similar tube incubated in the horizontal position is well colored in a few hours. In the following experiment tubes of dextrose bouillon covered by 5 cc. of vaseline were placed in a boiling water bath for 30 minutes at the end of which time the methylene blue in the control tube was colorless. The other sealed tube was cooled and inoculated by means of a capillary pipette through the layer of vaseline. There was good growth in both inoculated tubes.

Strain No.	Sugar.	Relation to air.	Incubated 7 days.	
			Titration.	pH
			per cent	
X	Dextrose.	Not sealed.	3.9	5.1
	"	Sealed.	2.1	6.2
Sterile methylene blue.	"	"	Remained colorless.	

The titratable acidity is expressed as per cent normal acid.

It is to be noted that the behavior of *Bacillus pyogenes* as regards fermentation of sugars in relation to oxygen tension appears at variance with that of other organisms, notably those of the colon group. The idea, possibly correct in the case of most organisms, is prevalent that facultative anaerobic organisms are able to satisfy their oxygen requirements by breaking down fermentable sugars and that in such cases fermentation is likely to be more vigorous or at least more apparent under anaerobic conditions than in the presence of free oxygen. Some strains of *Bacillus coli* may produce an alkaline reaction in the bulb and acid and gas in the closed arm of the fermentation tube containing saccharose bouillon. *Bacillus cloacæ* may react similarly in lactose bouillon. The alkalinity or lower acidity of the bulb, however, may not be due to diminished fermentative activity but to the simultaneous production of large amounts of alkali.

Growth in Milk.

One of the cardinal cultural characteristics of *Bacillus pyogenes* is its ability to coagulate milk and slowly digest the curd. If an indicator solution such as rosalic acid and china blue (Bronfenbrenner, 1918–19) or bromocresol purple (Clark and Lubs, 1917, *b*) is placed in the milk, the latter is seen to become acid and remain acid for at least 3 weeks during which time much of the coagulum disappears. The question arises as to whether the coagulation of milk is due to acid or to an enzyme.
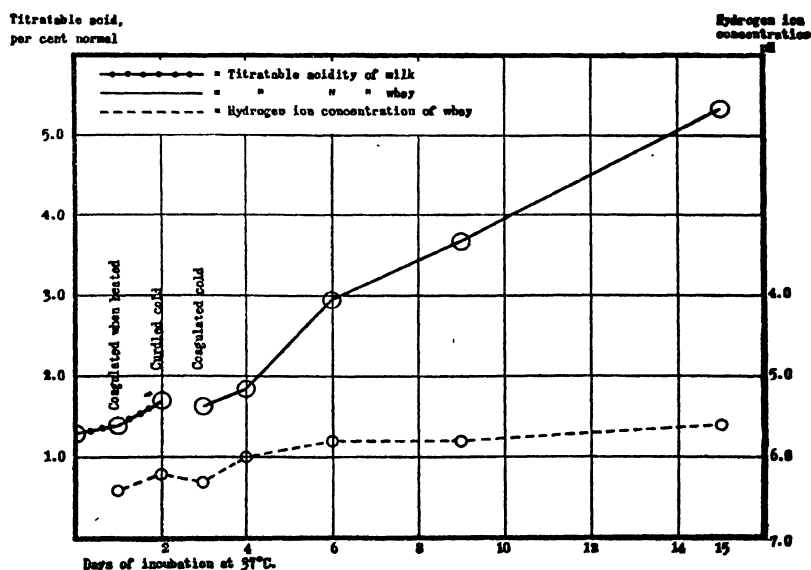
TEXT-FIG. 2. Titratable acidity and hydrogen ion concentration of fat-free milk after inoculation with *B. pyogenes.*

Seven tubes of fat-free milk were inoculated with *Bacillus pyogenes*. A tube was withdrawn for titration of total acidity and determination of hydrogen ion concentration[4] after incubation for 1, 2, 3, 4, 6, 9, and 15 days. The results are plotted in Text-fig. 2. The titratable acidity increased steadily. The hydrogen ion concentration of the whey

[4] Determinations of hydrogen ion concentration were made by the colorimetric method of Clark and Lubs (1917, *a*).

increased very little after the 6th day and reached a maximum of pH 5.6 in 15 days. The milk showed visible coagulation without application of heat on the 2nd day of incubation when the hydrogen ion concentration of the whey was pH 6.2 and the titratable acidity of the curdled milk was 1.7 per cent normal.

For comparison with these results tubes of milk were acidified with increasing amounts of 0.5 N acetic and hydrochloric acids. The results of titration of total acidity and determination of hydrogen ion concentration are plotted in Text-figs. 3 and 4.
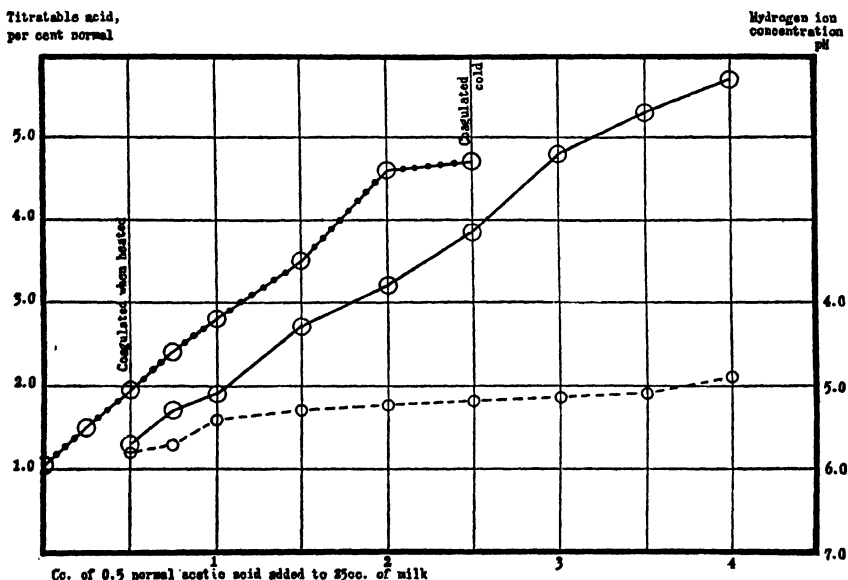
TEXT-FIG. 3. Total acidity and hydrogen ion concentration of fat-free milk after the tubes had been acidified with increasing amounts of acetic acid.

As was to be expected the hydrogen ion concentration showed more rapid increase with addition of hydrochloric acid than with similar amounts of acetic acid. With both the strong and the weak acid, however, the milk was coagulated without application of heat when it had reached a titratable acidity of 4.3 and 4.7 per cent normal respectively and the whey had a hydrogen ion concentration of pH 5.4 to 5.2. As was also to be expected the titratable acidity of the weak acid accompanying a hydrogen ion concentration sufficient to coagu-

late the milk was higher than that of the strong acid, the latter being more highly ionized. The difference is even more apparent in the titratable acidity of the whey.

In fermented bouillon plus 0.2 per cent of calcium chloride and 2.0 per cent of sodium caseinate *Bacillus pyogenes* produced a casein coagulum or sediment within 24 hours at 37°C. In the absence of calcium chloride no coagulation occurred. The same media gave similar results with rennet. These tests were made in media of four different reactions; namely, pH 6.2, 6.8, 7.1, and 7.7. After inoculation
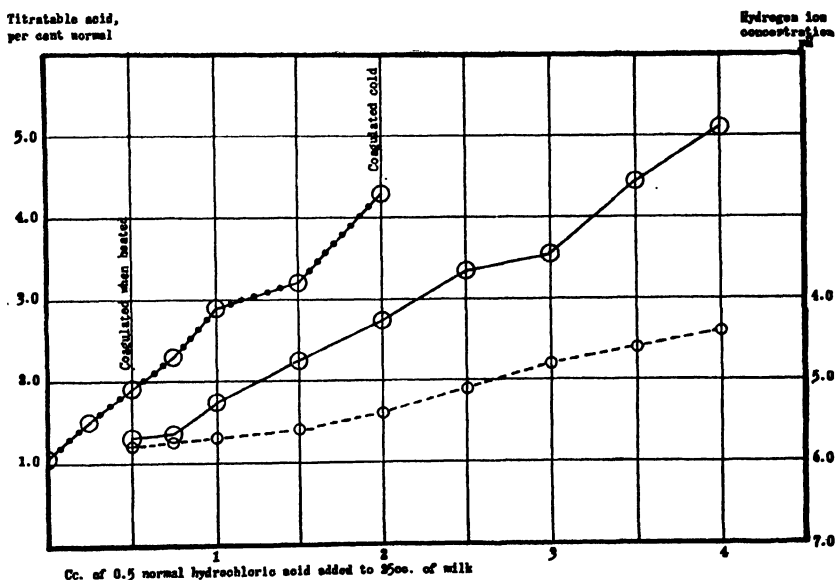
Text-Fig. 4. Total acidity and hydrogen ion concentration of fat-free milk after the tubes had been acidified with increasing amounts of hydrochloric acid.

with *Bacillus pyogenes* and incubation for 20 hours, during which time coagulation occurred in all of those containing calcium chloride, the reactions were pH 6.2, 6.5, 6.7, and 7.2 respectively. The sedimented coagulum in the last tube was the most voluminous and the supernatant fluid from this tube gave no further precipitate when acidified with acetic acid. In the tube with pH 6.2 the sediment was more compact than in the others but acidification of the supernatant fluid showed that some casein was still in solution. It has also been found

that when *Bacillus pyogenes* is grown in fermented bouillon, plain bouillon, serum bouillon, dextrose bouillon, ór dextrose serum bouillon plus sodium caseinate, the medium becomes very cloudy, almost opaque, in 24 hours and then clear again in another day or two. No sediment is formed or at most only a sediment of the organism itself. When the medium clears, however, the casein has disappeared and cannot be precipitated by acetic or nitric acid. Serum does not completely disappear and can be precipitated by nitric acid in cultures more than a week old. Apparently in the dextrose bouillon the casein is digested before the acidity rises sufficiently to precipitate it.

The preparation of casein calcium bouillon is attended with some difficulty. The method employed was as follows: To the sterile fermented bouillon add sufficient sterile 10 per cent calcium chloride to make a 0.2 per cent solution. Add sufficient sterile hydrochloric acid to dissolve the calcium phosphate which has been precipitated. At this point the medium reacts pH 6.2 to 6.4. Add sufficient 10 or 20 per cent sterile casein solution to make 2.0 per cent of casein in the bouillon. After the casein has been added the medium may be made alkaline if desired by the addition of sterile sodium hydrate. The calcium is apparently held by the casein so that it is not again precipitated as phosphate in the presence of a moderate amount of alkali. The preparation of this medium could probably be simplified by the use of calcium caseinate rather than sodium caseinate.

Since milk is coagulated by *Bacillus pyogenes* at a titratable acidity far below that required of hydrochloric or acetic acid and also at a much lower hydrogen ion concentration, and since soluble casein is coagulated in a neutral or slightly alkaline sugar-free medium, it appears that the coagulation of milk by *Bacillus pyogenes* is an enzyme rather than an acid coagulation. It is also to be noted that the casein is digested even in the presence of an excess of fermentable sugar. The process of digestion, however, seems to stop short of ammonia production since in a milk culture incubated for 3 weeks no increase in ammonia could be detected by Folin's method.

Immunological Study.

Four representative strains of *Bacillus pyogenes* were selected for the immunization of rabbits. Strain IV was selected because it showed slight morphological differences from the others. Strains VII, VIII, and X were selected because they were isolated from different lesions; *i.e.*, lungs, uterus, and a fetus, respectively. Rabbits

TABLE II.

Cross-Precipitation of Strains IV, VII, VIII, and X.

Strain No.	Serum No.	Dilutions.					Controls.	
		1:10	1:20	1:40	1:80	1:160	Normal rabbit serum, 1:10.	Salt solution.
IV	IV	++++	+++	++	++	+	−	−
	VII	++++	++	+	−	−	−	−
	VIII	-+	Sl.	−	−	−	−	−
	X	++++	++	+	−	−	−	−
VII	IV	++	++	+	Sl.	−	−	−
	VII	++++	++++	++	−	−	−	−
	VIII	+	−	−	−	−	−	−
	X	++++	++	+	Sl.	−	−	−
VIII	IV	++	+	Sl.	−	−	−	−
	VII	++++	+++	+	Sl.	−	−	−
	VIII	++	+	+	+	Sl.	−	−
	X	+++	++	++	+	+	−	−
X	IV	++	+	Sl.	−	−	−	−
	VII	+++	++	+	+	−	−	−
	VIII	+	Sl.	−	−	−	−	−
	X	++++	+++	++	+	−	−	−

In the tables + + + + indicates maximum precipitation; Sl., slight precipitation.

were given at first several series of subcutaneous and intravenous injections of killed cultures, and later increasing amounts of living bouillon cultures intravenously at intervals of about 1 week. The rabbits tolerated the injections well and though all eventually succumbed to *Bacillus pyogenes* infection noticeable lesions did not develop until after the immune serum had been secured.

TABLE III.

Precipitation of All Strains by Sera IV and X.

Strain No.	Serum No.	Dilutions.					Controls.	
		1:10	1:20	1:40	1:80	1:160	Normal rabbit serum, 1:10.	Salt solution.
I	IV	++	+	Sl.	—	+	—	—
	X	+++	++	++	+	Sl.	—	—
II	IV	+++	+++	+	Sl.	—	—	—
	X	++++	+++	++	+	+	—	—
III	IV	++	+	+	Sl.	—	—	—
	X	++++	++	+	—	—	—	—
IV	IV	++++	+++	++	++	+	—	—
	X	++++	++	+	—	—	—	—
V	IV	++	++	+	—	—	—	—
	X	++++	+++	++	±	—	—	—
VI	IV	+++	++	+	—	—	—	—
	X	++++	++++	+++	++	+	—	—
VII	IV	++	++	+	Sl.	—	—	—
	X	++++	++	+	"	—	—	—
VIII	IV	++	+	Sl.	—	—	—	—
	X	+++	++	++	+	+	—	—
IX	IV	++	+	Sl.	—	—	—	—
	X	+++	++	+	—	—	—	—
X	IV	++	+	Sl.	—	—	—	—
	X	++++	+++	++	+	—	—	—
XI	IV	+	Sl.	—	—	—	—	—
	X	++++	++	+	—	—	—	—
XII	IV	++	++	+	Sl.	—	—	—
	X	++++	++++	+++	++	+	—	—

The titration of agglutinins was unsatisfactory because all but one or two strains were agglutinated to a considerable extent by normal rabbit serum in dilutions of 1:100 or 1:1,000. There was relatively little agglutination in salt solution controls. By regarding as positive only those tubes in which agglutination was stronger than in the normal serum controls it was evident that the immune sera produced agglutination of the homologous strains and many others in dilutions of 1:800 to 1:3,200. However, the normal agglutinins were such a disturbing factor that it is considered unsafe to draw any conclusions as to the relationship of the various strains on the basis of agglutination.

More satisfactory results have been obtained by titrating the precipitins. The precipitinogen used in the titrations consisted of the clear supernatant fluid obtained by centrifuging bouillon and blood bouillon cultures after incubation for 1 month. During incubation the cultures were frequently shaken. The blood bouillon and the plain bouillon yielded equally good precipitinogen. The precipitin titer of the sera was much lower than the agglutination titer but there was no precipitation in the controls and the results were quite definite. In Table II are given the results of cross-precipitation of the four strains employed for immunization of rabbits. There is precipitation of all the strains by each of the sera. Except in the case of Strain VIII the precipitinogen of each strain is precipitated best by the homologous serum. Serum VIII was rather a weak serum probably due to the fact that the rabbit from which it was obtained could not be injected regularly because of its having "snuffles." No great diversity among the strains is revealed by the precipitin titration. Strain IV, however, does seem to stand a little apart from the other three. Serum IV and Serum X were therefore selected for titration with all the strains. The results are recorded in Table III. The precipitinogens of all the strains except No. IV show greater precipitation with Serum X than with Serum IV. It appears that Strain IV is slightly different from the others immunologically as well as morphologically.

Pathogenicity for Rabbits.

According to Berger (1908), Holth (1908), and others, rabbits are the most susceptible of the small laboratory animals to experimental infection with *Bacillus pyogenes*. Guinea pigs are less susceptible, and mice least so.

In addition to the four rabbits used for immunization. five others were injected intravenously with a single dose of 3 or 4 cc. of living bouillon culture, two with Strain IV and three with Strain X. Those immunized against Strains IV, VII, VIII, and X were repeatedly injected intravenously with these strains. Eight of the rabbits succumbed to the infection or were killed when in a badly crippled or moribund condition. The ninth may have died as a result of bleeding from the heart. There were no noticeable immediate symptoms following injections, no toxic symptoms, but usually a rise in temperature within 48 hours. Following this the rabbits appeared normal for 2 or 3 weeks. The first symptom of infection was often a progressive loss in weight followed by lameness or in four cases by paralysis. One of the paralyzed rabbits died during our absence from the laboratory and a complete autopsy was not obtained. The lesions found in the remaining seven rabbits may be classified as follows:

Lesions of bones in six rabbits (vertebræ three, femur four, rib one, tibia one).
Lesion of joint in one rabbit.
Lesions of muscles or tendons in three rabbits.
Lesion of lymph node in one rabbit.
Endocarditis in one rabbit.
·Pneumonia in one rabbit.
Kidney abscesses in one rabbit.

All the above lesions were studied histologically and culturally, and were found to be due to the organism injected. A summary of these results is given in Table IV.

Rabbit J was injected subcutaneously only with bouillon cultures of Strain X at three different times as follows:
1st day. Injection A, 0.2 cc.; Injection B, 1.0 cc.
7th day. Injection C, 0.2 cc.; Injection D, 0.4 cc.; Injection E, 0.6 cc.
10th day. Injection F, 0.2 cc.; Injection G, 0.4 cc.; Injection H, 0.6 cc.
12th day. Rabbit chloroformed. Abscesses removed and fixed in Zenker's fluid. The rabbit had lost about 100 gm. in weight but was otherwise apparently well. At autopsy the subcutaneous lesions were the only ones found.

The abscesses at the time of removal were therefore 2, 5, and 11 days old. The youngest abscesses appeared grossly as flat discs about the size of five cent pieces and on gross section appeared to be composed of a fibrous tissue infiltrated with a small amount of yellowish

translucent viscid pus, and surrounded by soft hemorrhagic edematous tissue. The largest and one of the oldest abscesses, B, was firm and nodular, about 1 cm., in diameter, composed of a thick capsule of dense fibrous tissue enclosing a thick creamy yellow somewhat viscid pus.

A stained section of one of the youngest abscesses, H (Fig. 10), is roughly oval, about 7 mm. long by 2 mm. broad. It lies in a loose areolar connective tissue with a layer of transversely cut muscle fibers within 1 mm. of one side of the abscess. There is no definite capsule. The abscess is bordered by a thin layer of fibrin and necrotic connective tissue. The connective tissue on all sides is infiltrated by polymorphonuclear leucocytes while the perimysium of the adjacent muscular tissue contains many eosinophilic cells. There are also some large mononuclear cells many of which are doubtless fibroblasts though some may be endothelial leucocytes. Some of the muscle fibers nearest the abscess are invaded by polymorphonuclear leucocytes and are undergoing heterolysis. The contents of the abscess are principally a mass of degenerating polymorphonuclear leucocytes. Scattered about are bits of collagenic fibers, each embedded in a mass of *B. pyogenes* as though the bacilli are growing on the collagenic fibers as a medium (Fig. 14). Very few bacilli are found elsewhere than clustered about these fibers. About these individual masses of bacilli there is always an area of compact necrosed cells with few visible nuclei and in one part these areas by confluence have formed the beginning of the characteristic central zone of the older abscesses.

The older abscesses, A and B, are spherical and surrounded by dense fibrous capsules, 2 to 3 mm. thick (Fig. 11). The abscess is differentiated into three fairly distinct zones. The central zone (Fig. 11, *a*) is a ragged granular mass of dead and disintegrating cells with few visible nuclei. Within this zone many bacilli are scattered about, but towards its periphery these are in masses only a few of which still contain a fragment of collagenic fiber. Apparently these fibers are digested by the proteolytic action of the bacilli. Outside the central zone no bacilli are found. In the second or intermediate zone (Fig. 11, *b*) most of the cells have deeply stained nuclei which, however, exhibit pycnosis, caryorrhexis, or caryolysis. The outer zone (Fig. 11, *c*) resembles the central zone in general appearance. There are few visible nuclei, but no bacilli. It is bordered, however, by large mononuclear macrophages laden with nuclear and other cell debris.

In sections the bacilli are best stained by Gram's method used according to Holth's (1908) directions. Stirling's aniline gentian violet containing 1 or 2 per cent by weight of the dry stain has given us excellent results. To show the association of bacilli with collagenic fibers we have obtained excellent preparations with the following stains.

Orth's lithium-carmine, 20 minutes.
Acid alcohol, 3 minutes.
Stirling's aniline gentian violet, 10 seconds.

TABLE IV.

Rabbits Inoculated with B. pyogenes.

Rabbit.	Sex.	Strain No. Injection.	Maximum temperature. °C.	Weight variation. gm.	Localizations.	Result.
A	M.	IV. Repeated subcutaneous and intravenous injections for immunization.	40	1,890–2,355	Yellow spots in liver; hemorrhagic foci in lungs. (Incomplete autopsy.)	Died suddenly during night after being bled, 39th day after first injection of living culture.
B	"	VII. Repeated subcutaneous and intravenous injections for immunization.	41	1,650–1,865–1,380	Left femur; both kidneys; heart valve; epicardium.	Died after period of weakness 97th day after first injection of living culture.
C	"	VIII. Repeated subcutaneous and intravenous injections for immunization.	40.8	1,850–1,944–1,705	Muscle abscess in left quadriceps extensor femoris muscle. (Recurrent attacks of snuffles.)	Chloroformed 4½ mos. after first injection of living culture.
D	"	X. Repeated subcutaneous and intravenous injections for immunization.	40.2	1,650–2,010	3rd and 4th lumbar vertebre; abscess surrounding and invading rib.	Paralysis of both hind legs. Chloroformed 5 mos. after first injection of living culture.
E	"	IV. 3 cc. intravenously.	39.8	1,960–2,125–1,945	(Incomplete autopsy.)	Paralysis of both hind legs and bladder. Died suddenly on 34th day after injection.

	F.					
F		X. 3 cc. intravenously.	41.2	1,935-1,980-1,040	Left femur.	Died 27 days after injection.
G	M.	IV. 4 "	40.5	1,645-1,935-1,470	1st thoracic vertebra; right knee; both shoulder joints (periarticular abscesses).	Paralysis of left hind leg. Chloroformed on 81st day after injection.
H	"	X. 4 "	41.4	1,835-1,895-1,240	Right femur; tendon of left gastrocnemius muscle; left instep; lungs.	Died on 13th day after injection.
I	"	X. 3 "	39.8	1,815-1,910-1,240	1st, 2nd, and 3rd lumbar vertebræ; crest of right tibia; trochanter and gluteus minimus muscle of right femur; right zygomatic fossa; right biceps brachii muscle; left cubital gland.	Paralysis of both hind legs. Chloroformed on 64th day after injection.
J	"	X. Multiple subcutaneous injections.	39.4	3,580-3,415	Subcutaneous abscesses at points of injection.	Chloroformed 11th day after first injection.

Wash in water.
Lugol's iodine solution, 30 seconds.
Wash in water. Blot.
Decolorize in absolute alcohol.
Wash in water.
Mallory's aniline blue and orange G, 20 minutes.
Wash in water.
Decolorize and dehydrate in 95 per cent and absolute alcohol.
Xylol. Mount in balsam.

By the above method *B. pyogenes* is stained purple, collagenic fibers bright blue, leucocytes and tissue cell nuclei red or deep orange, blood, fibrin, and muscle yellow.

Of the lesions resulting from the intravenous injection of cultures a few deserve brief description. The most frequently produced lesions were those of the bones. Such lesions have been reported by Berger (1908), Holth (1908), and Koske (1906). Their protocols show that they encountered paralyses also in experimentally infected animals. Koske, unable to find lesions in the cords of paralyzed pigs, considered it possible that a specific neurotoxin might be involved but was unable to obtain toxic effects with filtered cultures. He does not mention examining the vertebræ of these animals. Four times we encountered paralyses in injected rabbits. In the three that were thoroughly autopsied were found lesions on the ventral floor of the spinal canal. These lesions were not visible from the ventral side of the spinal column and in fact could not be found until the cord had been removed. There were then found abscesses exerting pressure against the ventral side of the cord. In no case was the dura penetrated nor were the meninges infected. In the case of Rabbit G an abscess lay between the dura and the vertebral periosteum without invading the body of the vertebra. In Rabbits D and I were found intervertebral abscesses obliterating the intervertebral cartilages, invading the bodies of the vertebræ, and eroding the bone with more or less destruction of the floor of the spinal canal (Fig. 12).

In a stained section there is found proliferation of connective tissue about the abscess. The center of the focus is composed of a mass of disintegrated cells and nuclei which appear to have been small mononuclear cells rather than polymorphonuclear leucocytes, differing in this respect from the abscesses in the subcutis and other soft parts. In the proliferating fibrous tissue bordering and sometimes sur-

rounding the abscesses in the vertebræ are many large mononuclear cells (Fig. 15). Stained by Gram's method many of these large cells are seen to be filled with *B. pyogenes* (Fig. 16). Holth regards these cells as young fibroblasts rather than endothelial cells. In the necrotic center of the abscess bacilli lie scattered about.

The lesion here described is much like the "*grösseren Knoten*" described by Holth and found by him in various parts of the body—lungs, subcutis, peritoneum, etc. He describes masses of "*Rundzellen*" in the center of the abscess undergoing necrosis and disintegration, an intermediate zone of tissue resembling the round cell masses of smaller abscesses, and an outer capsule of connective tissue interrupted by small masses of round cells. As the tumor grows the round cell masses fuse and the process of disintegration advances. He then describes the bacilli scattered about in the center of the abscess and within what he regards as large connective tissue cells of the capsule. He does not mention the presence of polymorphonuclear leucocytes in the abscess, whereas we found them to predominate in abscesses of soft parts. It is to be noted, however, that because of pycnosis these cells often resemble round cells. It is to be noted also that in the subcutaneous abscesses described above no bacilli were found in the cells of the capsule, possibly because the abscesses studied by us were not old enough.

Lesions in the long bones—femur and tibia (Fig. 13)—were fundamentally like those in the bodies of the vertebræ, modified by the tissues encountered. The predominating cells of the reaction resembled plasma cells. In places there was marked proliferation of connective tissue. In places the bacilli appeared to grow freely among the cells of the bone marrow. Occasionally bone lacunæ were seen filled with masses of bacilli. There was always erosion of the bone making it quite porous. In two cases the femur broke under very little strain as the rabbits were being tied out for autopsy. A nodular encapsulated abscess enveloped the rib of Rabbit D eroding it from without. The abscess may have been subperiosteal in origin.

The knee of Rabbit G contained a glairy viscid pus and one of the joint surfaces was eroded.

In Rabbits C, H, and I true myositis and tendinitis were encountered. In these cases the muscle fibers had been completely heterolyzed or digested and the muscle converted into a closed sac of viscid

glairy pus within the epimysium (Fig. 9). ' There were present in the pus and perimysium plasma cells, endothelial leucocytes, neutrophilic polymorphonuclear leucocytes, and, especially conspicuous, eosinophilic leucocytes. Bacilli were abundant in all but the oldest lesions.

The diseased mitral valve of Rabbit B was encrusted with masses of *Bacillus pyogenes*, leucocytes, and necrotic tissue. The tissue of the valve was largely fibrin.

In the pneumonic lung of Rabbit H masses of bacilli were found beneath the pulmonary pleura. It appeared that growth had started from bacilli lodged in the pleural capillaries. The alveoli were filled with blood, fibrin, and desquamated epithelium.

The kidney abscesses of Rabbit B were in the form of pyramids with bases at the cortex. Around the borders of the abscesses the glomeruli and the capillaries of the interstitial tissue were plugged with leucocytes and masses of bacilli. The interlobular arteries were similarly plugged.

Resemblance to Other Organisms. Classification.

' The close resemblance of *Bacillus pyogenes* to streptococci has been mentioned above. This is especially true of some strains when grown on certain media, a resemblance so close that the bacteriologist working with milk or animal diseases must be on his guard not to confuse them. *Bacillus pyogenes* produces laking of a suspension of blood or blood corpuscles in salt solution as do the hemolytic streptococci of human origin (Brown, 1920). Its limiting hydrogen ion concentration in dextrose bouillon would also mislead one to place it among the human streptococci (Avery and Cullen, 1919). On the other hand, *Bacillus pyogenes* liquefies coagulated blood serum and is distinctly diphtheroid at times. Morphologically, therefore, it may be one of the diphthero-streptococci now and then described. Such organisms, are occasionally isolated from human lesions, especially pneumonias and though *Bacillus pyogenes* has never been identified in man, it would be well for bacteriologists to keep it in mind. Since it is found in several species of animals and is widely disseminated in milk it may be that there are rare cases of human infection.

There is also found in animals a group of small Gram-positive organisms closely resembling *Bacillus pyogenes* morphologically and culturally. We have studied two such strains from the livers of calves and one from the lung of a hog. The strains from calves were apparently alike, and had the same fermentation reactions as *Bacillus pyogenes*. They were cultivated with greater difficulty than the latter, however. They grew best under partially anaerobic conditions and would not grow in serum-free media. There was no apparent growth in milk. Gelatin and coagulated serum were not liquefied and growth on the latter was scarcely visible. There was very little laking of horse blood in agar plates. A rabbit was repeatedly injected intravenously with several cubic centimeters of serum bouillon cultures of one of these strains with no ill effects. Serum from this rabbit precipitated precipitinogens of both of these strains in dilution of 1:80 or 1:160 but produced no precipitation of the precipitinogens of strains of *Bacillus pyogenes*. Neither did *Bacillus pyogenes* antisera precipitate precipitinogens of these two strains.

A strain from a hog also resembled *Bacillus pyogenes* morphologically. It differed from *Bacillus pyogenes* and the other two strains just described in that it fermented salicin. It resembled the two strains from calves in failing to liquefy gelatin or coagulated serum but grew much better than those strains. No visible change was produced in milk but sufficient acid was produced so that when the culture tube was placed in boiling water the milk was coagulated. An indefinite zone of hemolysis was produced in the blood agar plate.

Glage (1913) points out a certain resemblance of *Bacillus pyogenes* to the bacillus of swine erysipelas. Morphology, growth in gelatin, and the lesions produced in hogs and small experimental animals serve to differentiate the two.

Dunkel (1908) thought that *Bacillus pyogenes* could be transformed into *Bacillus pseudotuberculosis ovis* by animal passage and therefore regarded the two as of the same species. The two are certainly not alike when studied as isolated from their respective hosts. The dry colonies described as characteristic of *Bacillus pseudotuberculosis*, pigmented colonies on coagulated blood serum, and failure to produce change in milk are not at all like *Bacillus pyogenes*. There may be some morphological resemblance.

Priewe (1911) and Glage (1913) have claimed that *Bacillus pyogenes* belongs to the influenza bacillus group for the following reasons: hemoglobinophilic habit, form, size, non-motility, lack of sporest abundance in green pus, growth at high temperature only, and sligh-virulence for small laboratory animals. Priewe claimed that a *Ba, cillus pyogenes* antiserum agglutinated *Bacillus influenzæ*. We have not tried to repeat the latter observation. However, we do regard it as well established that *Bacillus pyogenes* is Gram-positive and that it is not hemoglobinophilic. The other characters enumerated by the above authors are common to so many and dissimilar organisms that they are of little value as evidence of relationship of these two organisms. *Bacillus pyogenes* does not have the foul odor character-istic of *Bacillus influenzæ* and the two do not produce similar lesions in rabbits. Grips (1903), Grips, Glage, and Nieberle (1904), and Priewe (1911) have regarded swine-plague as due primarily to *Bacillus pyogenes* with *Bacillus suisepticus* as a secondary invader. They regard the primary infection as a "*Tierinfluenza*." The views of these authors have not been accepted by others. Olt (1904) has criticized them thoroughly but asserts that many of the lesions char-ácteristic of chronic swine-plague are due to *Bacillus pyogenes*.

The forms assumed by *Bacillus pyogenes* bear a striking resemblance to those of *Asterococcus mycoides* described by Borrel, Dujardin-Beau-metz, Jeantet, and Jouan (1910) as the cause of bovine pleuropneu-monia. The latter organism is, however, much the smaller. The work of the authors mentioned is almost wholly morphological and the result of staining by Gram's method is not mentioned. Buchanan (1918), however, places *Asterococcus* in the Gram-negative subtribe Hemophilinæ. The other genus of this subtribe is *Hemophilus*, of which the type species is *Hemophilus influenzæ*, the influenza bacillus of Pfeiffer. Since Buchanan and Murray (1916) describe *Bacillus pyogenes* as a member of the hemophilic or influenza group they pre-sumably classify it also in the subtribe Hemophilinæ where it can hardly belong in view of its being Gram-positive. Preisz (1906) regarded *Bacillus pyogenes* as one of the "*Corynebakterien*." We are more inclined to place it in the genus *Corynebacterium* as defined in the Preliminary Report of the Committee of the Society of American Bacteriologists on Characterization and Classification of Bacterial Types (1917) than in the so called influenza group.

SUMMARY.

Bacillus pyogenes is probably quite common in this country, as it is known to be in Europe.

A careful study of twelve strains from cattle and one from a hog has disclosed the following characteristics which have not been reported or have been in dispute.

Bacillus pyogenes is Gram-positive and pleomorphic, producing forms ranging from short chains of streptococcoid elements to branching filaments.

It is hemolytic, producing the beta type of hemolysis in blood agar. It is not hemoglobinophilic, though its growth is greatly favored by some higher protein material such as egg albumin, serum, or blood.

It ferments xylose in addition to the substances previously reported.

The coagulation of milk by *Bacillus pyogenes* is primarily an enzyme coagulation and the subsequent digestion of the curd takes place in an acid medium.

The intravenous injection of rabbits was invariably fatal. The lesions most commonly developed were those of the bones. Paralysis was frequently produced, and in each case was caused by lesions in the vertebræ exerting pressure against the ventral columns of the spinal cord. Muscle abscesses were also frequently produced.

The authors regard the organism as belonging to the *Corynebacteria* rather than to the influenza group.

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EXPLANATION OF PLATES.

PLATE 14.

FIG. 1. A horse blood agar plate culture of *B. pyogenes* X after incubation for 48 hours. The large round hemolyzed area was produced by a few particles of saponin.

PLATE 15.

Gram stains. Magnification X 1,000.

FIG. 2. The bacillary form of Strain X.

FIG. 3. The fusiform form of Strain IV.

FIG. 4. The fusiform form of Strain X.

FIG. 5. The filamentous or branching form of Strain IV.

FIG. 6. The diphtheroid form of Strain X showing a few buds.

FIG. 7. The streptococcoid form of Strain IV.

FIG. 8. The streptococcoid form of Strain X.

PLATE 16.

FIG. 9. Rabbit H. Abscess in tendinous end of gastrocnemius muscle at *a*. Above the abscess is a section of the distal end of the flexor digitalis pedis sublimis muscle. Eosin and methylene blue stain. × 10.

FIG. 10. Rabbit J. Subcutaneous abscess H, 48 hours after injection. The capsule and zonal arrangement of the abscess not yet developed. Gram and aniline blue stain. × 5.

FIG. 11. Rabbit J. Subcutaneous abscess A, 11 days after injection. A very thick fibrous capsule (*d*) about the abscess and the three zones (*a*, *b*, *c*) of the abscess described in the text are shown. Eosin and methylene blue stain. × 5.

FIG. 12. Rabbit D. Abscess occupying the body of the third lumbar vertebra at *a*, the spinous process and dorsum of the spinal foramen having been removed. Only a small bony fragment of the floor of the spinal canal remains at *c*. Eosin and methylene blue stain. × 5.

FIG. 13. Rabbit I. Abscess in crest of tibia at *a*. Transverse section. Eosin and methylene blue stain. × 10.

FIG. 14. Rabbit J. A field from the center of the abscess shown in Fig. 10, showing the minute bacilli clustered about remnants of connective tissue fibers. Gram and aniline blue stain. × 430.

FIG. 15. Rabbit D. A field from the region marked *b* in Fig. 12, showing large mononuclear cells probably on the border of an abscess. Eosin and methylene blue stain. × 430.

FIG. 16. Rabbit D. A field from the same region as Fig. 15, showing the large mononuclear cells filled with *B. pyogenes*. Gram stain. × 430.

(Brown and Orcutt: *Bacillus pyogenes.*)

PLATE 16.
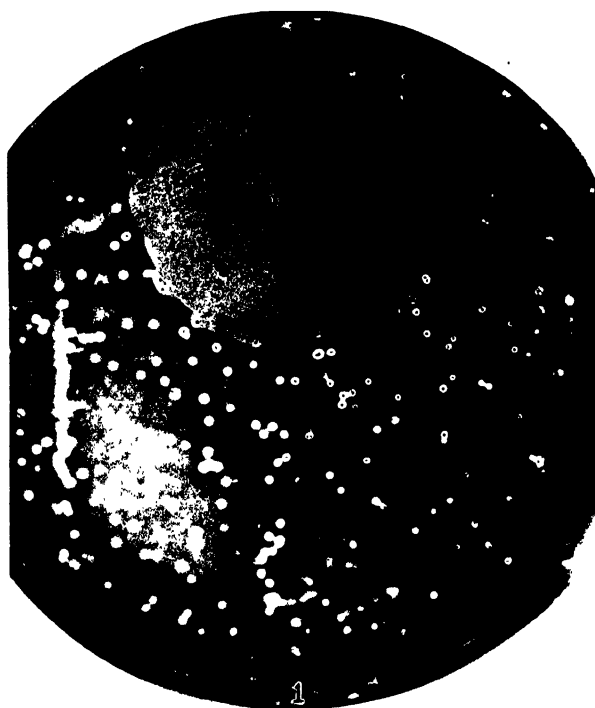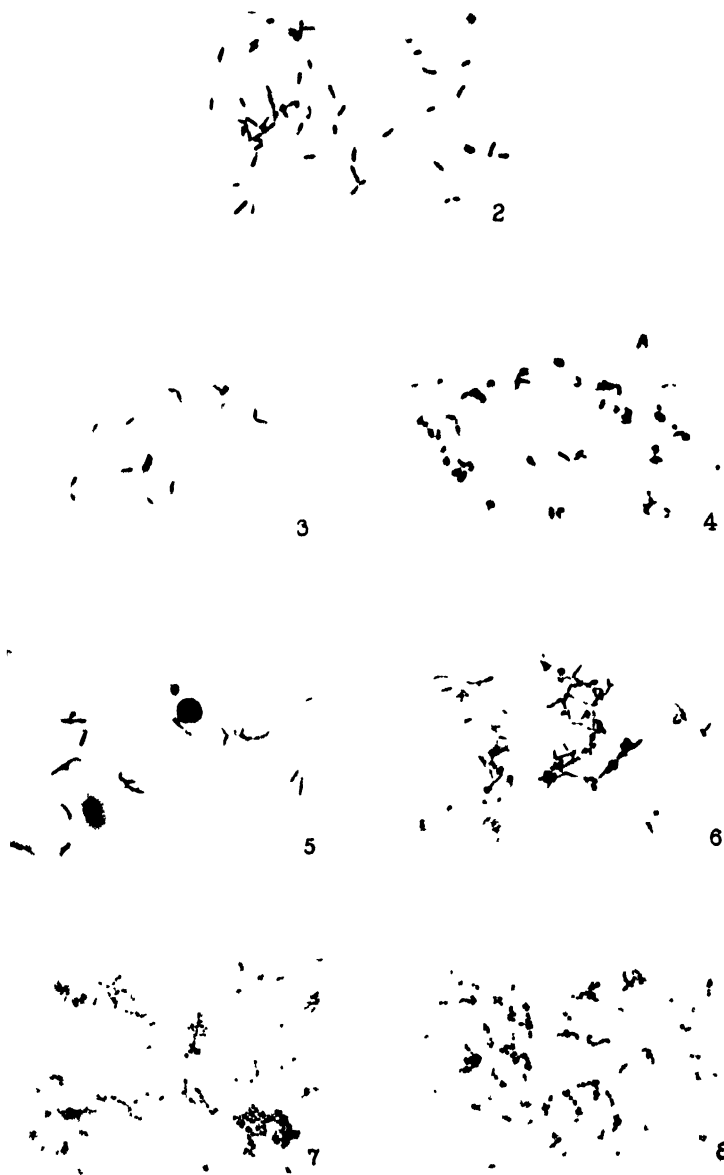
(Brown and Orcutt: *Bacillus pyogenes.*)
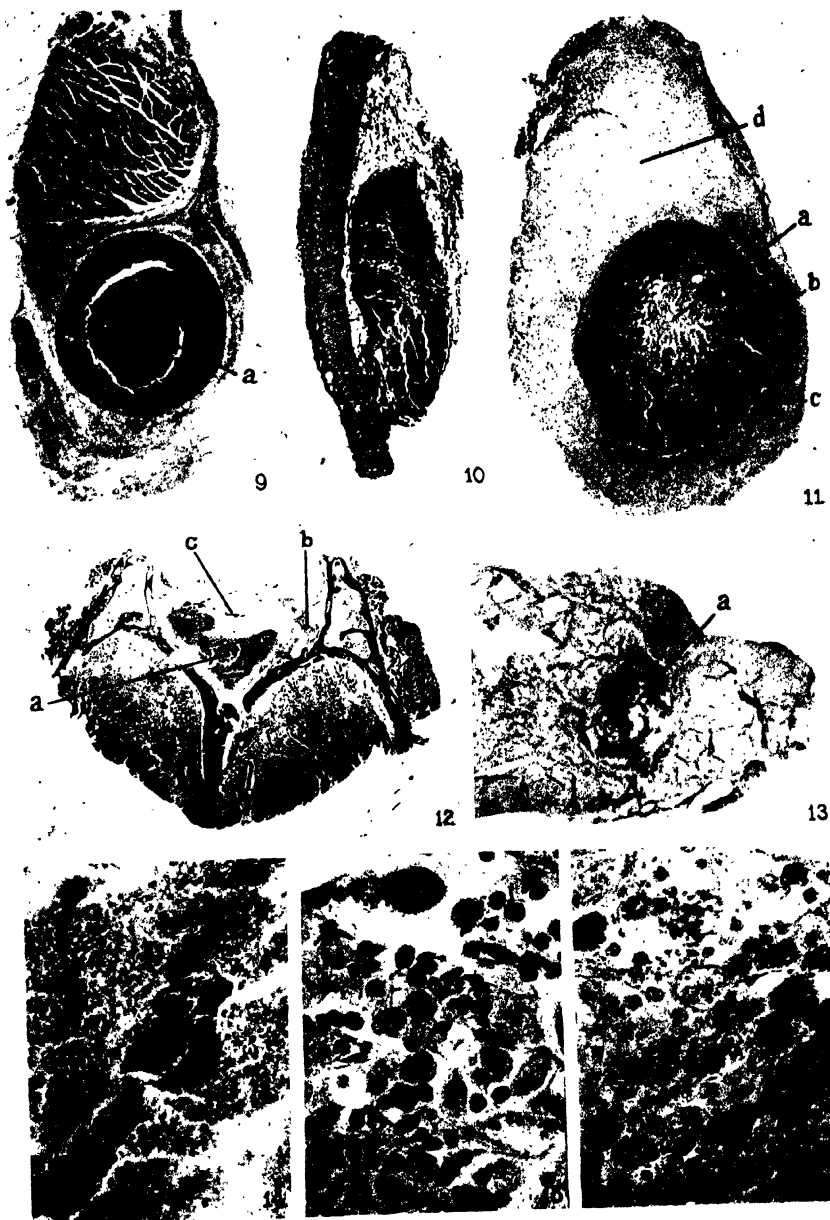
INFLUENCE OF VARIATIONS OF MEDIA ON ACID PRODUCTION BY STREPTOCOCCI.

BY F. S. JONES, V.M.D.

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In an attempt to differentiate species of organisms closely resembling each other, the fermentation of carbohydrates and other substances plays an important part. When the fermentation characters of streptococci were first studied indicators such as litmus were employed which revealed only qualitative changes. Later quantitative determinations of acid by titration against solutions of alkali came into use.

More recently Clark and Lubs[1] have employed the hydrogen ion concentration method as an aid in the differentiation of closely allied species of organisms. Several have applied it for determination of acid production by streptococci. The advocates of the newer method point out that it is more accurate than titration since it indicates only free acids. The term "limiting or final hydrogen ion concentration" has come into use, since it indicates the maximum acidity or alkalinity produced by a given organism. Thus Avery and Cullen[2] have shown that human streptococci grown in veal infusion bouillon containing 1 per cent dextrose attained a limiting hydrogen ion concentration of 5.0 to 5.2. The bovine group produced more acid, 4.3 to 4.5. Ayers, Johnson, and Davis[3] some time previously had grown human hemolytic streptococci in a desiccated yeast-peptone-dextrose medium, one part of each per 100 parts of water, and found that the bulk of their strains reached a final hydrogen ion concentration of 5.4 to 6.0; 5.6 seems to have been the average. Smillie,[4] who used 1 per cent dextrose in fermented veal infusion bouillon, records the figures pH 5.1 to 5.4 for a few human strains. Brown[5] observed a limiting acidity of pH 5.1 to 5.4 for human streptococci grown in plain bouillon containing 1 per cent dextrose.

[1] Clark, W. M., and Lubs, H. A., *J. Bacteriol.*, 1917, ii, 1.
[2] Avery, O. T., and Cullen, G. E., *J. Exp. Med.*, 1919, xxix, 215.
[3] Ayers, S. H., Johnson, W. T., and Davis, B. J., *J. Infect. Dis.*, 1918, xxiii, 290.
[4] Smillie, W. G., *J. Infect. Dis.*, 1917, xx, 45.
[5] Brown, J. H., *J. Exp. Med.*, 1920, xxxi, 35.

Fennel and Fisher[6] record the acid limit of *Streptococcus hæmolyticus* as pH 4.5. Whether these figures include both the human and bovine varieties is not stated.

Since considerable variation in acid production had been observed by various workers it seemed possible that the differences might be the result of variations in the media. Broadhurst[7] brought out the difference in the amount of titratable acid produced by streptococci grown in broth prepared from meat and meat extract. Streptococci grown in the former media produced two or three times as much acid as those grown in meat extract media. Many have observed the marked increase in the amount of titratable acid when more than 1 per cent of peptone was used in sugar broths. The writer has frequently noted that streptococci grown in fermented bouillon ·containing 1 per cent dextrose to which sterile serum had been added invariably produced more titratable acid than cultures in the same media which did not contain serum. A small number of tests also showed that the hydrogen ion concentration was often greater in the dextrose serum broth. The results, then, were not readily explicable on the assumption that the buffer activity of the serum was responsible for the increase in the acidity. It seemed desirable to note the effect of variations of the medium upon acid production.

Veal infusion was prepared from the flesh of a calf 1 day old. It is customary in this laboratory to add two parts of water to one part of the ground flesh. The infusion was divided. To one portion 1 per cent of peptone (Fairchild's) and 0.5 per cent of sodium chloride were added and the reaction was adjusted to + 0.8 (pH 7.6). The remainder of the infusion was strained and inoculated with a young culture of *Bacillus coli* and permitted to ferment at 38° C. for 18 hours. After straining through cotton and gauze, the usual quantity of peptone and salt was added to the larger portion of the filtrate. The reaction was adjusted to + 0.8 (pH 7.6). To a smaller portion of the fermented infusion 2 per cent of peptone and the usual amount of sodium chloride were added. This then provided three types of bouillon prepared from the same materials, unfermented broth and fermented bouillon containing 1 and 2 per cent of peptone respec-

[6] Fennel, E. A., and Fisher, M. B., *J. Infect. Dis.*, 1919, xxv, 444.

[7] Broadhurst, J., *J. Infect. Dis.*, 1913, xiii, 404.

tively. The broths were tubed in amounts of 13 cc. After sterilization under pressure, 1 cc. of a sterile 13 per cent solution of dextrose was added to each. The columns of liquid in the tubes ranged from 6 to 6.5 cm. in height. To half of the tubes of fermented and half of the tubes of unfermented bouillon, 0.5 cc. of sterile horse serum per tube was added. The 2 per cent peptone-dextrose fermented broth was used without other additions.

All cultures employed in the experiment produced hemolysis (beta) in agar plates containing 8 per cent of defibrinated horse blood. All the human streptococci[8] had been grown on artificial media for 2 or more years. All had been isolated from diseased conditions. The bovine strains were isolated by the writer. Five were from cases of mastitis. Two were found in market milk, but were identical in all respects with mastitis streptococci. The equine streptococci, with one exception, were isolated from the nasal mucosa and pharynx of horses suffering from influenza. Equine Streptococcus H.A. 2 was cultivated from an abscess. The low acid-producing streptococci were isolated from market milk. Recent investigations[9] have shown that these may be carried in small numbers in apparently normal udders. Strains M.J. 1 and M. 53 were isolated in 1917. The others were first cultivated in the summer of 1918.

Each tube was inoculated with 0.1 cc. of an 18 hour broth culture. All were incubated at 38°C. for 10 days, when hydrogen ion determinations[10] and titrations against 0.05 N sodium hydroxide were made. Maximum growth was always obtained in the media containing serum. The plain and fermented bouillon cultures were about as vigorous as those observed in the 2 per cent peptone medium. The low acid-producing streptococci from milk grew poorly in media without serum. Serum frequently changes the character of the growth. Strains which grow only at the bottom of tubes in the plain broth frequently produce a marked turbidity throughout the serum medium.

[8] The writer is indebted to Dr. J. Howard Brown, of this Department, for Cultures 32, S.H., S. 8, and 40. Dr. O. T. Avery, of the Hospital of The Rockefeller Institute, supplied Cultures 1, 20, and 24.

[9] Jones, F. S., *J. Exp. Med.*, 1920, xxxi, 347.

[10] Dr. P. E. Howe, of this Department, prepared the standards and indicators for this experiment.

In Tables I to IV the acid production by the different groups of streptococci is given. The figures under the titration columns represent the actual acidity reached in the tubes.

TABLE I.

*Acid Production by Human Streptococci in Plain and Fermented Broths.**

Strain No.	Fermented bouillon.		Fermented bouillon and serum.		Fermented bouillon containing 2 per cent peptone.		Plain bouillon.		Plain bouillon and serum.	
	pH	Titration.	pH	Titration.	pH	Titration.	pH	Titration.	pH	Titration.
		per cent		*per cent*		*per cent*		*per cent*		*per cent*
32	5.5	3.6	5.2	4.2	5.7	4.5	5.1	4.2	5.0	4.8
S.H.	5.5	4.2	5.2	4.5	5.5	5.0	5.2	4.0	4.9	4.6
S. 8	5.8	3.9	5.2	4.1	6.0	3.9	5.6	3.2	4.9	4.2
40	5.2	4.2	5.0	4.6	5.4	4.9	5.1	4.1	5.0	4.7
1	5.8	3.5	5.2	4.2	5.8	3.9	5.5	3.3	5.0	4.4
20	5.7	3.1	5.2	4.0	5.7	3.9	5.2	3.2	5.1	4.4
24	5.7	3.1	5.1	4.2	5.7	4.3	5.1	3.7	4.9	4.5

* All the media contained 1 per cent dextrose.

TABLE II.

Acid Production by Bovine Streptococci in Plain and Fermented Broths.

Strain No.	Fermented bouillon.		Fermented bouillon and serum.		Fermented bouillon containing 2 per cent peptone.		Plain bouillon.		Plain bouillon and serum.	
	pH	Titration.	pH	Titration.	pH	Titration.	pH	Titration.	pH	Titration.
		per cent		*per cent*		*per cent*		*per cent*		*per cent*
B.M. 1	4.6	6.1	4.6	6.2	4.6	7.0	4.6	5.7	4.6	5.9
C. 59	4.8	5.7	4.8	6.2	5.0	7.0	4.6	6.2	4.6	6.1
B.M. 24	4.9	5.4	4.8	6.1	4.9	6.7	4.6	5.8	4.5	5.7
M. 26	4.8	5.5	4.7	6.3	4.8	7.1	4.6	5.8	4.5	5.7
" 43	4.7	6.0	4.6	6.5	4.7	7.2	4.6	5.9	4.6	5.9
C. 53	4.8	5.3	4.7	6.0	4.8	6.8	4.6	5.5	4.5	5.9
" 67C	4.8	6.2	4.8	6.5	4.8	7.4	4.6	6.0	4.6	5.9

Curves (Text-figs. 1 and 2) constructed from averages of Tables I to IV reveal considerable differences in acid formation in the various media. This is particularly true in the instance of the human and low acid-producing streptococci from milk. It will be observed that

these organisms grown in media low in nutritive material (fermented and plain broth), even though the media contain sufficient carbohydrate, fail to produce as much acid as when a richer medium is employed. The addition of serum, then, not only increases the titratable acid but the ionized acid as well. Thus in the richest medium

TABLE III.

Acid Production by Equine Streptococci in Plain and Fermented Broths.

Strain No.	Fermented bouillon.		Fermented bouillon and serum.		Fermented bouillon containing 2 per cent peptone.		Plain bouillon.		Plain bouillon and serum.	
	pH	Titration.	pH	Titration.	pH	Titration.	pH	Titration.	pH	Titration.
		per cent		*per cent*		*per cent*		*per cent*		*per cent*
In. 22	5.1	4.4	5.5	4.4	5.0	5.5	4.9	4.4	5.1	4.6
H.A. 2	5.1	4.3	5.5	3.2	5.1	5.3	4.7	4.4	5.0	4.6
In. 49	5.1	4.5	5.3	5.0	5.1	5.7	4.9	4.4	5.1	4.8
" 14	5.1	3.9	5.4	4.4	5.0	4.8	4.9	3.9	5.1	4.6
" 2	5.1	4.7	5.4	4.2	5.1	5.6	4.9	4.6	5.1	4.5

TABLE IV.

Low Acid-Producing Streptococci from Milk.

Strain No.	Fermented bouillon.		Fermented bouillon and serum.		Fermented bouillon containing 2 per cent peptone.		Plain bouillon.		Plain bouillon and serum.	
	pH	Titration.	pH	Titration.	pH	Titration.	pH	Titration.	pH	Titration.
		per cent		*per cent*		*per cent*		*per cent*		*per cent*
M.J. 1	6.3	2.6	5.4	4.1	6.5	3.1	5.2	4.0	5.2	4.0
M. 53	6.3	2.7	5.8	3.5	6.3	2.4	5.8	3.0	5.2	4.1
B.M. 30	6.6	2.0	5.2	4.1	5.9	3.7	5.1	4.0	5.1	4.3
" 22	5.9	3.1	5.1	4.6	6.3	3.7	5.5	3.4	5.1	4.6
" 60	6.3	2.4	5.1	4.4	6.3	3.2	5.5	3.9	5.1	4.4

(dextrose bouillon and serum) the maximum acid production is reached. The sharpest differences are brought out by the low acid-producing milk streptococci; here the greatest increases in acid formation are seen in the serum media. The fermented broth containing 2 per cent peptone apparently offers for the human and low acid-producing streptococci no more favorable media for acid production than that

containing 1 per cent peptone. One would expect the titratable acid to increase on account of the great buffer activity of peptone.

The bovine streptococci follow a somewhat similar curve. Apparently organisms of this type are able to utilize carbohydrate to
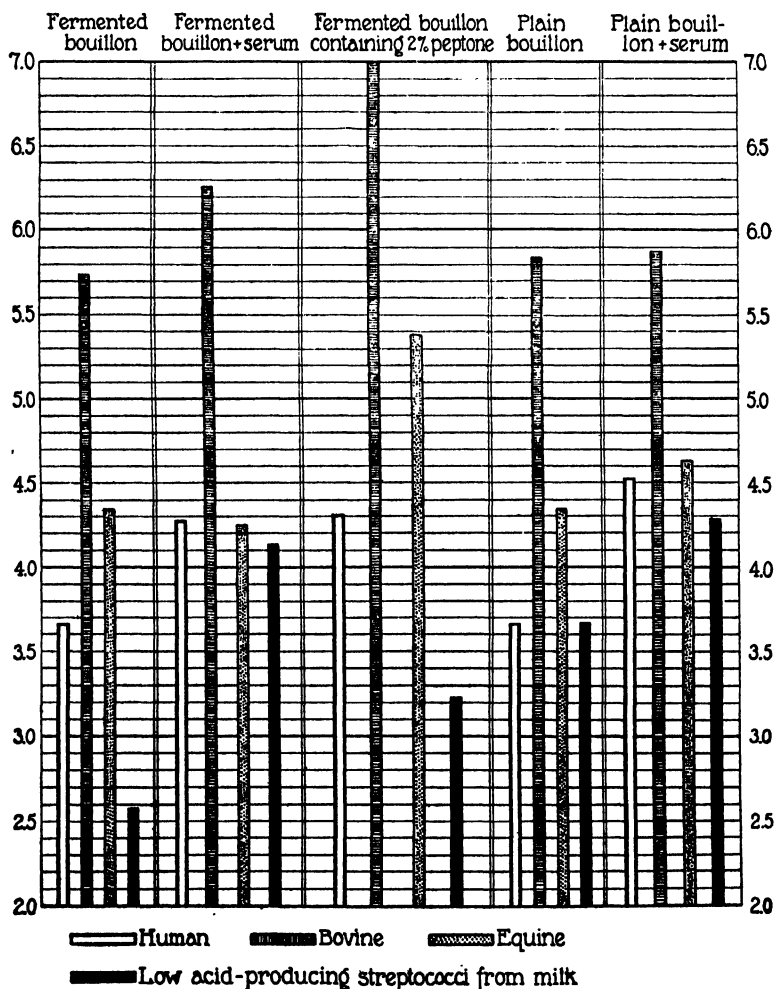
TEXT-FIG. 1. Titratable acidity produced by human, bovine, and equine streptococci and low acid-producing streptococci from milk when grown in various media.

a greater degree in media of lower nutritive value. The titratable acidity varies over a considerable latitude; the buffer effect of the 2 per cent peptone is marked. On the whole, there is more acid produced in the serum media.

The reverse is true of the equine streptococci. The organisms apparently produce more acid in media which do not contain serum. Broth containing 2 per cent peptone affords a medium equally as good for acid production as either of the other bouillons to which serum was not added. Two possible explanations for the failure of the
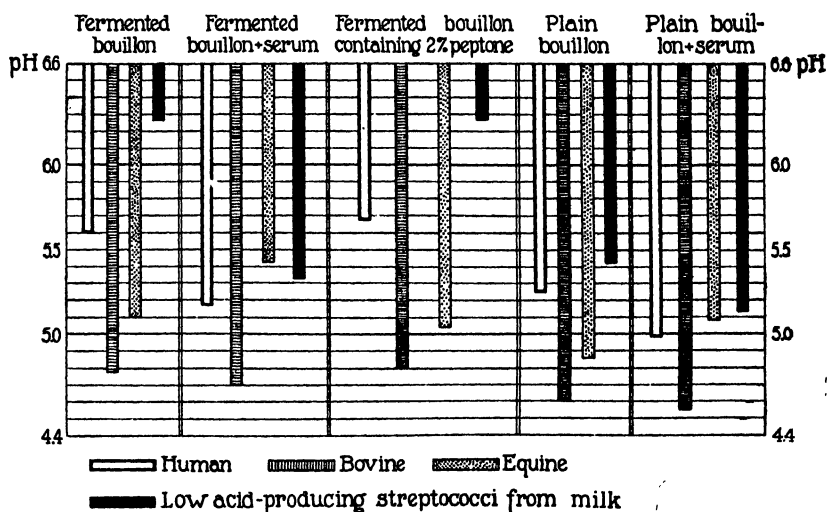
TEXT-FIG. 2. Limiting or final hydrogen ion concentration of human, bovine, and equine streptococci and low acid-producing streptococci from milk when grown in various media.

serum to increase the production of acid suggest themselves. The equine streptococci may not require serum; peptone and meat extracts may be sufficient for all purposes. On the other hand, normal horse serum may contain some substance antagonistic to growth. It is admitted that all strains grew well in the serum media.

The experiment was repeated with the same cultures, but with broths made from the flesh of different calves. When bouillon prepared from the flesh of a calf 6 weeks old was employed much the same results were obtained.

DISCUSSION.

The results obtained readily show that the limiting hydrogen ion concentration may be influenced by differences in the media. The question naturally arises as to the final or limiting hydrogen ion concentration reached by an organism. From present knowledge it may be defined as the acid production in a given medium which finally limits the growth of the organism. That such figures vary over a considerable latitude must be admitted, since human streptococci in one medium may produce acid to the value of pH 5.6 and in another to pH 4.9. Avery and Cullen's figures represent the maximum acid production for human (pH 5.0 to 5.2) and bovine (pH 4.3 to 4.5) streptococci in a medium most favorable for growth. Ayers, Johnson, and Davis' figures (5.5 to 6.0), on the other hand, are given as the final hydrogen ion concentration in a medium of low nutritive value for pathogenic streptococci. While these data were being gathered together the paper of H. Jones[11] appeared. He obtained even greater variations than those recorded in the preceding protocols. He showed that a culture of *Streptococcus hæmolyticus* when grown in glucose broth produced acid to the value of pH 5.11, but when ascitic fluid was added to the bouillon an acidity of pH 4.63 was reached. The increase in acidity failed to vary with an increase in the amount of enriching material. The same limiting hydrogen ion concentration was reached in media to which varying amounts (3 drops to 3 cc.) of ascitic fluid had been added. H. Jones also observed that the initial reaction of the media may influence the limiting hydrogen ion concentration. Thus in concluding it is pointed out that the limiting hydrogen ion concentration of an organism should be defined in terms of media composition, the initial reaction, and other conditions which may favor or hinder abundant growth.

Clark and Lubs[12] noted that methyl red may be destroyed in a short time by active cultures of *Bacillus coli*. Others had observed the same for nitrifying bacteria. Certain streptococci, especially those from sour milk, exhibit this activity to a marked degree while in the active growth phase. The indicator begins to fade within 5 or 10 minutes and at the end of an hour is completely decolorized.

[11] Jones, Horry, *J. Infect. Dis.*, 1920, xxvi, 160.
[12] Clark, W. M., and Lubs, H. A., *J. Bacteriol.*, 1917, ii, 191.

The advocates of the exclusive application of the hydrogen ion concentration method to bacteriological study have frequently pointed out the unreliability of titration. In studying the tables and charts it will be observed that the differences in titratable acidity are clear-cut. Even the addition of 2 per cent peptone with its buffer effect still reveals great differences in the amount of acid formed by the different groups of organisms. From these experiments one is inclined to believe that titration is equally as satisfactory as the newer method for the study of the fermentative activity of streptococci.

The greatest differences in acid production are brought out in a relatively unfavorable medium, such as dextrose fermented broth containing 1 per cent peptone.

SUMMARY.

The results of variations in acid production in 1 per cent dextrose fermented and unfermented veal broth modified by the addition of 4 per cent of horse serum or 2 per cent of peptone have been recorded. Human streptococci and a group of low acid-producing streptococci from milk produce less acid in the simpler broths (fermented and unfermented). 2 per cent peptone fails to increase the amount of acid produced by these two groups.

The bovine streptococci act much the same as those of human origin. The equine streptococci apparently do not require serum in addition to carbohydrate since they tend to produce less acid in serum media.

The following figures indicate the average minimum and maximum acid production of the various streptococci under the conditions set forth in the experiment.

Human: pH 4.97 to 5.66; titratable acidity 4.51 to 3.66.
Bovine: pH 4.56 to 4.77; titratable acidity 7.0 to 5.74.
Equine: pH 4.86 to 5.42; titratable acidity 5.38 to 4.24.
Low acid-producing streptococci from milk: pH 6.28 to 5.14; titratable acidity 2.56 to 4.28.

STUDIES ON BACILLUS MURISEPTICUS, OR THE ROTLAUF BACILLUS, ISOLATED FROM SWINE IN THE UNITED STATES.

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PLATE 24.

No essential differences have been detected between the mouse septicemia bacillus described by Koch (1) and the rotlauf or swine erysipelas bacillus subsequently discovered by Loeffler (2). The studies to be reported here are on organisms obtained from swine which, if they had been isolated in Europe, would undoubtedly have been called rotlauf bacilli, but since swine erysipelas is a disease that has not thus far been recognized in the United States, and since Koch's organism was the first to be described, they are called mouse septicemia bacilli, or *Bacillus murisepticus*.

There are reports that organisms of this group have been isolated three times in this country. Smith (3) in 1885 obtained a culture from the spleen of a pig dead of hog-cholera, and Moore (4) in 1888 obtained a culture from the spleen of another pig affected with the same disease. Smith (5) in 1894 again obtained a culture from the cervical lymph node of a pig that presumably had hog-cholera. This last paper is of special interest, for in it we find the first description of scurvy in guinea pigs and a demonstration that such animals may be susceptible to organisms to which the normal guinea pig is immune. In addition, there is a discussion of the relation of feeding to disease, a subject about which even today we know very little, but the importance of which we are beginning to realize.

These organisms are not difficult to isolate, and if they were at all common in the bodies of pigs with hog-cholera they would have been reported more frequently. In Europe, and especially in Germany, there have appeared many reports concerning their presence in swine. Koch (1) obtained the mouse septicemia bacillus from mice that he had inoculated with putrid material. How many times he found it and how frequently it has subsequently been obtained in this way cannot be determined from the literature. In 1882 Loeffler (2) found similar organisms in the bodies of swine affected with rotlauf, rouget, or swine

erysipelas. They were present in great numbers not only in lesions of the skin but generally distributed in the body, and he regarded them as the cause of the disease. Pasteur and Thuillier (6) had previously studied rouget and described as the cause an organism which from their description is difficult to identify. That they cultivated the organism described by Loeffler is shown by the observations of Schütz (7) and Smith (8), both of whom found the mouse septicemia bacillus in the vaccine prepared by Pasteur for protecting against rouget. The organisms found by Loeffler resembled Koch's bacillus in their morphology, growth in gelatin, and in being virulent for pigeons, white and gray mice, but not for field mice. Olt (9) quotes Loeffler as finding that animals which had recovered from an infection produced by the injection of rotlauf bacilli were immune to *B. murisepticus* and *vice versa*. This power of one organism to immunize against the other has repeatedly been confirmed, and Rosenbach (10) and others have shown that rotlauf serum will protect against the mouse septicemia bacilli as well as against the organisms obtained from swine.

Rosenbach (10) and Preisz (11) found the mouse septicemia bacillus to be slightly larger and to grow more rapidly in gelatin than the organisms obtained from swine. Rickmann (12), on the other hand, studied over 100 cultures obtained from swine with rotlauf and found considerable variation in their morphology, so that he could not differentiate some of them from the one culture of mouse septicemia he had, which was the one used by Rosenbach. Stickdorn (13) found that the frequent transfer of a virulent rotlauf culture or its passage through a series of mice or pigeons changed not only the virulence but the character of the growth in gelatin, and Smith (5) found that the same cultural character was markedly influenced by the reaction of the medium.

When injected immediately after its isolation the rotlauf bacillus may be virulent for swine, but after it has been on culture media for any length of time or after it has been passed through some of the experimental animals it loses this virulence and then resembles *B. murisepticus* in that it fails to cause disease when injected into pigs.

Organisms that could not be differentiated from those found in swine with rotlauf have been isolated from chickens by Schipp (14) and from sheep with arthritis by Poels (15). In continental Europe bacilli of swine erysipelas have also been found in swine that were apparently normal. Bauermeister (16) inoculated fifteen mice with the secretion from the tonsils of fourteen pigs. From five of the mice he obtained cultures of what he called rotlauf bacilli. Olt (9) also found these organisms in normal swine, but he does not give the number of animals examined or the number of cases that were positive. Van Velzen (17) studied the tonsils of eleven normal pigs and from three obtained rotlauf bacilli. Pitt (18) inoculated mice with the secretion from the tonsils of 50 pigs and with the secretion from the glands at the ileocecal valve of 66 pigs. 56 per cent of these swine showed rotlauf bacilli in the tonsils and 40 per cent in the glands at the ileocecal valve.

That these organisms are the cause of rotlauf seems to be generally accepted. The fact that they are usually present in great numbers and the encouraging results obtained by vaccination are in favor of this view. It is, however, unusual to find such a large percentage of normal animals that are carriers of organisms that are the primary agent of a disease. Olt (9) has suggested that these bacteria may gain entrance to the body through lesions produced by intestinal parasites and then become virulent; Smith (5) emphasizes the relation of feeding to an invasion of the body. There is, furthermore, the possibility that the organisms found in normal swine differ from those found in rotlauf. Proof of any of these hypotheses is lacking and the facts as we now know them suggest that these organisms may be secondary invaders. There are diseases in which secondary invaders are almost invariably present and at times, due to their number or the ease with which they are isolated, are regarded as the primary agents of a disease. In some cases it is only by the most careful work and the use of special methods that the primary agent of the disease is recognized. The object of this paper is to call attention to the fact that these organisms occur in the United States where the disease of swine erysipelas is unknown, to give the cultural reactions of the organisms isolated, to record some observations on the pathology of the disease produced in experimental animals, especially the mouse, and finally to raise the question as to whether these bacilli really are the primary agent of rotlauf in swine.

Isolation of Cultures from Swine.

In a study of the tonsils of pigs that had been inoculated with hog-cholera virus some of the exudate from an ulcer on the tonsil was injected into mice. These mice died in a few days and from them were isolated organisms that corresponded to the mouse septicemia bacillus. Following this first observation, material from the tonsils of all the swine that came to autopsy was injected into mice, and when these animals died, cultures were made from the spleen and heart's blood and films from the lungs were stained by Gram's method and examined for the intracellular Gram-positive rods, which are characteristic of *Bacillus murisepticus*.

Sixteen swine, all infected with hog-cholera virus, have been examined in this way and from the tonsils of five of these bacilli have been isolated which morphologically and culturally are mouse septicemia bacilli. The organisms were apparently localized in the tonsils, for they were not found in cultures made by transferring pea-sized bits of liver, spleen, and kidney to agar slants.

Four of the positive swine were from the same litter and the source of the fifth one is questionable, so that it appears that the infection is restricted. On the farm from which these animals came there is no record of any diseases among pigs, and before inoculation the animals were apparently well.

These organisms may have some relation to the ulcers that are so commonly present on the tonsils of pigs with hog-cholera, as four of the five positive cases showed ulcers, whereas only four of eleven negative cases showed them. It is just as possible, however, that a variety of organisms may be associated with these ulcers, as they are probably due to invasion by bacteria of a lesion produced by the hog-cholera virus. Bacilli of the swine-plague group were often present and killed the inoculated mice before the mouse septicemia bacilli had time to invade the bodies of these animals. It would be of interest to know how common and how widespread the mouse septicemia bacilli are in the United States, and if such a study is made it would be well to immunize part of the mice used for their isolation by the injection of anti-swine-plague serum as was done by Van Velzen (17).

Cultural Characters.

The ease with which mouse septicemia bacilli are identified probably accounts for the meager description of their cultural reactions. The morphology has been well described, as has the growth on agar and in gelatin, but this is as far as most descriptions go. It therefore seemed worth while to gather together the recorded reactions and to fill in the most obvious gaps. The five cultures isolated recently and the one culture isolated by Smith (5) in 1894 have been used, and all have been found to give the same results.

Morphology.—These organisms are non-motile, Gram-positive rods, varying considerably in length and diameter, the variation depending

upon the source from which preparations are made. In films from mice, dead after inoculation with a pure culture, they appear as very slender, straight or slightly curved rods from 1.5 to 2 μ in length. They may appear free or are grouped in a characteristic manner in large cells with an indefinite nucleus. From the surface of Loeffler serum slants and in gelatin incubated at 37°C. they appear as straight or slightly curved rods somewhat thicker than those found in the tissues of mice. In bouillon, and especially on agar slants, this increase in size is more marked. The organisms may measure up to 4 μ in length and show a decided tendency to curve and form clumps of interlacing bacilli. Branching forms have not been found in films made from cultures on a variety of media incubated up to 5 days. In sections of tissues stained by the Gram-Weigert method the organisms often have a beaded appearance, but in films stained by Gram's method, using Stirling's gentian violet and decolorizing with alcohol, the organisms stain uniformly.

Agar.—Growth appears in 24 hours in the form of fine, translucent, slightly gray colonies. It is very scanty, but becomes more abundant if serum or defibrinated blood is added to the medium.

Gelatin.—Stab cultures show the characteristic "test-tube brush" growth described by Loeffler (19) which has been serviceable in identifying these organisms. Petri and Maassen (20) obtained the same type of growth in a semisolid medium at incubator temperatures, but I have been unable to confirm this. Smith (5) noted that when the gelatin was acid this characteristic growth did not occur. After some weeks incubation gelatin stabs show a finger-like depression which is not found in uninoculated tubes that are incubated the same length of time. If this is due to a softening and evaporation of the gelatin, as has been stated, it is evident that it proceeds at a very slow rate and that it only occurs in the presence of oxygen. Gelatin cultures that have been incubated at 37°C. for 30 days will still harden when placed in the refrigerator.

Bouillon.—In 24 hours there is a uniform turbidity and when shaken a characteristic cloud-like effect is produced that has been considered of diagnostic value.

Blood Serum.—Several writers state that these organisms do not grow on this medium, while Poels (15) states that it grows but does

not liquefy the serum, this being one of the differential points between the rotlauf bacillus and *Bacillus pyogenes*. All of my cultures have formed a scanty growth on Loeffler serum slants and after 30 days incubation there was no evidence of liquefaction.

Milk.—All writers agree that no visible change is produced in this medium. Moore (4) found microscopic evidence of vigorous growth of the one strain he studied, while Schipp (14) found no evidence of growth of the three strains with which he worked. All of my cultures have failed to produce acid in milk, yet all of them show microscopic evidence of growth. As will be noted below, these organisms attack lactose in bouillon, but not in milk.

Ammonia.—6 day cultures in fermented bouillon failed to show any appreciable amount of ammonia.

Hydrogen Sulfide.—Petri and Maassen (20) found this gas in their cultures, and in swine dying of rotlauf they found sulfmethemoglobin, but they did not find sulfmethemoglobin in experimental animals inoculated with pure cultures. All six of my cultures blackened lead acetate in peptone agar in 24 hours.

Indole.—Schipp (14) has the only reference that I have found to indole production, his three cultures all giving a negative test. All six of my strains in fermented bouillon cultures 5 days old gave a negative test with Ehrlich's aldehyde as did a typhoid culture and the uninoculated medium. A colon culture gave a positive reaction.

Phenol.—No reference has been found on phenol production. Three of my cultures were grown for 10 days, each in 40 cc. of fermented bouillon contained in 100 cc. Erlenmeyer flasks. They were then distilled with steam and the distillate was tested with bromine water and Millon's reagent. Reactions for phenol were not obtained.

Hemolysins.—Van Nederveen (21) found that his culture did not hemolyze swine blood agar in plates, or the blood of cattle, rabbits, or pigeons in bouillon. The six cultures of the present study all produced a distinct zone of hemolysis around the deeper colonies in veal infusion agar plates containing about 10 per cent of sterile defibrinated horse blood. The same blood in bouillon was not hemolyzed.

Fermentation of Carbohydrates.—The literature gives very little information about this important phase of the bacterial activity of these organisms, and that found is not in agreement. Fermi (22) states that

the rotlauf bacillus forms acid from starch but that it does not possess a diastatic enzyme. Petri and Maassen (20) found that the growth of their rotlauf cultures was increased when dextrose, lactose, saccharose, or dextrin was added to the medium. Smith (5) studied one culture and found that acid but no gas was formed in bouillon containing dextrose or lactose, while no acid was formed in bouillon containing saccharose. Schipp (14) studied three cultures and found that acid was formed in a litmus peptone solution containing lactose or saccharose, while in the same medium containing dextrose the litmus was decolorized but was not reddened.

Preliminary tests with fermentation tubes containing fermented bouillon plus 1 per cent of either dextrose, lactose, or saccharose

TABLE I.

Carbohydrate.	Initial reaction.		Length of incubation.	Final reaction.		
	Acidity.	pH		Acidity.		pH
	per cent		*days*	*per cent*		
Dextrose......................	0.7	7.5	5	2.7–3.1		6.1–5.8
Lactose.......................	0.9	7.5	7	2.4–3.1		6.4–6.0
Arabinose.....................	1.3	7.3	7	1.6–1.8		6.7–6.6
"	1.3		10	1.8–2.1		
" fermentation tubes.....	1.1		7	Bulb.	Branch.	
				1.7–2.1	1.1–1.5	

showed that these organisms did not form gas, but produced acid from the first two carbohydrates and that somewhat more acid was produced in the presence of oxygen than in its absence. In saccharose bouillon there was no acid or alkali formed. The great majority of the tests has been made in ordinary test-tubes containing 13.5 cc. of fermented bouillon to which 1.5 cc. of a previously autoclaved 10 per cent solution of the carbohydrate in distilled water was added. The tubes were steamed and incubated to insure sterility and were then inoculated from young bouillon cultures and titrated after from 5 to 7 days incubation. When acid was produced hydrogen ion determinations were also made, but when the carbohydrate was not acted upon this was not done. In Table I are given the extremes of reaction produced by the six cultures when acid was produced, the actual amount

of acid formed being the difference between the initial and final reactions of the medium.

It is evident that dextrose and lactose are attacked. The small amount of acid formed in the arabinose medium might indicate that there were impurities present which were acted upon while the carbohydrate itself was not attacked. The lot of arabinose used was the best obtainable and had been used for some time with satisfactory results in differentiating the hog-cholera bacillus from the other paratyphoids. If the mouse septicemia bacilli attack it at all they do so very slowly.

Fermented bouillon cultures containing the following carbohydrates have also been titrated and no acid has been found.

Xylose.	Inulin.
Dulcitol.	Salicin.
Maltose.	Dextrin.
Mannitol.	Starch.
Saccharose.	Glycerol.

Reduction.—The one attempt to study the reducing powers of these organisms was a failure as the presence of 1 per cent of either rosalic acid or methyl red apparently inhibited growth.

Pathogenicity.

Considerable work has been done on the virulence of the mouse septicemia bacilli for a variety of animals. Koch (1) found that they produced a fatal disease in white and gray mice, while the field mouse was immune. Loeffler (2) found that pigeons and sparrows were as susceptible as mice, and that frogs, salamanders, chickens, dogs, cats, and white rats were immune. In rabbits an erysipelas-like infection of the ears and a loss in weight followed an intravenous injection of the bacilli, but the animals usually recovered and later were immune. The virulence of the organisms isolated from pigs with rotlauf corresponds to that of the mouse septicemia bacillus except that very freshly isolated cultures may infect swine. This virulence is soon lost and the cultures are then the same as the organism isolated by Koch.

The strains which I have isolated are all virulent for white mice in as small amounts as 0.001 cc. of a 24 hour bouillon culture. The mice

are usually dead by the 3rd day. The culture isolated by Smith 26 years ago is also virulent for mice in the same amounts, but the animals die from 2 to 3 days later. These cultures when injected subcutaneously into pigeons cause death in about 4 days. Intravenous injection into rabbits causes a marked edema of the ear on the side of the injection and at times the opposite ear is also involved. The animals are very quiet, show a rise in temperature to around 41°C., and lose from 150 to 250 gm. in weight. Only one of the cultures will kill rabbits when injected either intravenously or subcutaneously. Whereas in mice and pigeons after death the organisms are abundant in all the organs and in the blood stream, in rabbits they are very scarce. They are rarely found in films, and in cultures made from bits of the various organs, or from several drops of blood, growth in agar slants occurs only in the condensation water.

Three pigs have been inoculated with the cultures isolated from the tonsils. One was given an intravenous injection of 1 cc. of a 24 hour bouillon culture. There was a slight rise in temperature without any signs of illness. The skin was normal and the appetite undiminished. The pig was then inoculated with hog-cholera virus and at autopsy cultures were made from the liver, spleen, and kidney, with negative results.

Two pigs were inoculated with another strain, one receiving 5 cc. of a 24 hour bouillon culture intramuscularly and the other 4 cc. intraperitoneally. Neither pig showed any effects from the inoculation. These results correspond to those obtained by Smith (5) and Moore (4) with the mouse septicemia bacilli which they isolated in this country.

One of the striking features of films made from mice and pigeons is the great number of organisms that is found in leucocytes. Koch (1) first called attention to this, and it has since been used as one of the means of identifying these organisms. Curiously enough, I have found no statement as to the type of cells in which these bacteria are found. From the drawings of Koch (1) and Moore (4) one might assume that they are polymorphonuclear leucocytes, since some of the cells show two nuclei. In the literature they are called leucocytes without further qualification.

Several years ago I made some experiments with an old stock culture of the mouse septicemia bacillus, one of the objects being to determine in which type of cell it occurred. After a subcutaneous injection of about 0.01 cc. of a 24 hour bouillon culture mice died on the 4th or 5th day. A number of inoculated mice were killed at intervals, and films, sections, and cultures made from the various organs. Cultures showed that the organisms were generally distributed at the end of 2½ days, but they were so scarce that they could not be found in films. On the 4th day, when the animals appeared to be sick, the bacilli could be found in films, especially in those from the lungs. Except when present in great numbers, they were always intracellular, and the only type of cell in which they could be found was the endothelial leucocyte (Figs. 1 and 2). Sections of the various organs showed that the bacilli at this stage were in the endothelial cells lining the veins and capillaries and also in endothelial cells free in the blood stream (Figs. 4 and 5). They were not found in endothelial cells lining the arteries or the heart. As the time of death approached the organisms were present in enormous numbers, filling the endothelial cells and crowding the nucleus to one side so that the cell resembled a sac containing a culture of the bacilli. When films were made these sacs were ruptured and the organisms were set free.

In pigeons the process was apparently the same except that the organisms were found generally distributed about 24 hours earlier than in the mouse. They were in the endothelial cells lining the veins and capillaries, and the type of cell in the blood containing them was apparently an endothelial leucocyte (Fig. 3). In both the mouse and the pigeon the organisms were at no time found in polymorphonuclear leucocytes.

Endothelial cells with only a few bacteria soon show evidences of injury. The cytoplasm contains small vacuoles and the nucleus when stained with a modified Romanowsky stain has a more reddish tinge than the nucleus of the same type of cell that is free from bacteria. As the bacilli become more numerous larger vacuoles appear in the cytoplasm and the nucleus stains distinctly red and shows evidences of disintegration.

The disease in the mouse appears, then, to be associated with an intracellular process. The organisms are taken up and instead of

being destroyed are able to multiply and finally to kill the cell. Some of these infected endothelial cells probably break away from the walls of the vessels and float free in the blood stream. Rous and Jones (23) have called attention to the fact that phagocytosed bacteria may be protected against immune substances in the body fluids. There are other instances of the multiplication of bacteria in cells. The leprosy bacillus may be found in the endothelial cells of blood vessels and the tubercle bacillus and *Treponema pallidum* are usually intracellular. Smith (24) has called attention to the localization of *Bacillus abortus* in the chorionic epithelial cells of cattle, and Tyzzer (25) to a disease of Japanese waltzing mice in which the organisms are found in the liver cells and cells of the intestinal mucosa. It is worthy of note that most of the diseases in which the organisms are found in cells are more or less slowly progressive, while the disease in the mouse produced by *Bacillus murisepticus* is acute.

SUMMARY AND CONCLUSIONS.

In the United States organisms, which culturally are mouse septicemia or swine erysipelas bacilli, have been isolated from the tonsils of five of sixteen pigs examined. These pigs all had hog-cholera, but it is probable that the bacilli were in the tonsils before they were infected with hog-cholera, and there is no evidence that they played any part in the disease. The distribution of the infection seemed to be restricted as most of the pigs from which the bacilli were obtained came from one litter. As we do not have clinical rotlauf, or swine erysipelas, in this country, as these organisms, in Europe, have been found in a large percentage of apparently normal swine, and as the disease is produced with difficulty by the injection of cultures, the question may be raised whether they are not secondary invaders rather than the primary cause of the disease with which they have been associated, or else whether the resistance of swine on the European continent does not differ from that of our breeds as a result of differences in foods.

It is possible that the mouse septicemia bacilli found in this country may differ culturally from those present in animals with swine erysipelas. With this in mind, the carbohydrate reactions, as well as

other cultural characters not necessary for the identification of the bacilli isolated, have been studied.

The disease produced by the injection of these bacilli into mice and pigeons has been studied and shown to be largely an intracellular process. The organisms are taken up by the endothelial cells lining the veins and capillaries; there they multiply and soon kill the cells. It has also been shown that the only type of cell in the blood stream which contains bacteria is the endothelial leucocyte, and the probabilities are that the free phagocytes have been detached from the lining of the vessels. The disease is acute, and the indications are that in the cells the bacilli find a favorable medium for their growth. While phagocytosis may in general be an immune reaction, in this case it appears to favor the parasite rather than the host.

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EXPLANATION OF PLATE 24.

Fig. 1. Film from the heart's blood of a mouse killed 3 days after the subcutaneous injection of 0.01 cc. of a bouillon culture of freshly isolated *B. murisepticus*. Bacilli in an endothelial cell. × 1,000.

Fig. 2. Film from the lung of the same mouse. Bacilli in two endothelial cells. × 1,000.

Fig. 3. Film from the lung of a pigeon dead after a subcutaneous injection of 0.01 cc. of a bouillon culture of freshly isolated *B. murisepticus*. Bacilli in a mononuclear cell which is probably an endothelial leucocyte. Free organisms from ruptured cells. × 1,000.

Figs. 4 and 5. Sections of the kidney of a mouse showing *B. murisepticus* in the endothelial cells of a vein. × 1,000.

(TenBroeck: *Bacillus murisepticus.*)
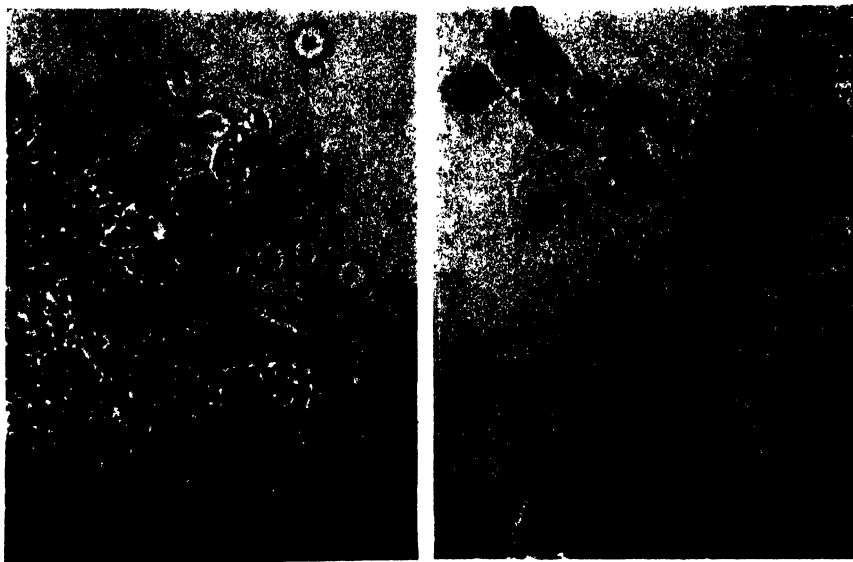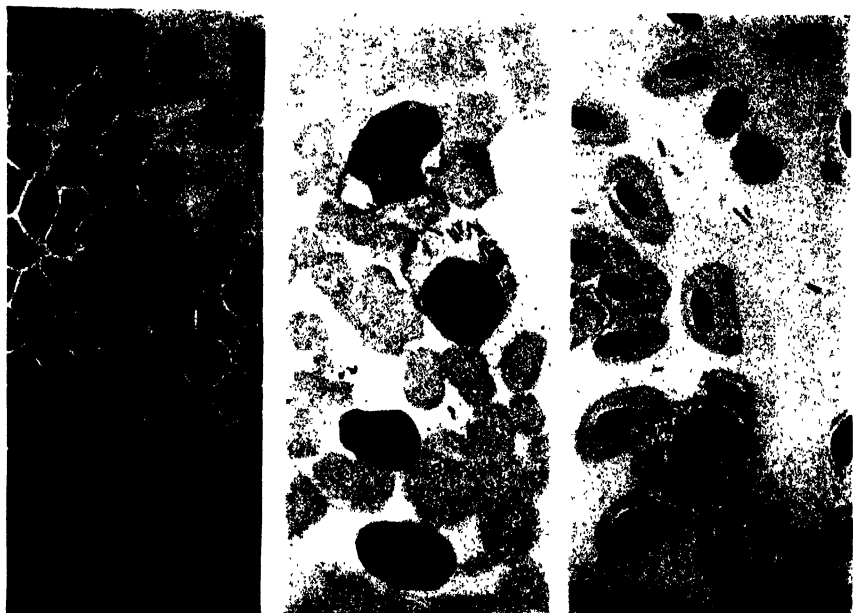
EFFECTS OF ENZYMES IN SERUM ON CARBOHYDRATES AND THEIR RELATION TO BACTERIOLOGICAL TECHNIQUE.

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While studying the carbohydrate reactions of the mouse septicemia bacillus (1) a source of error was detected which apparently has not been appreciated by some bacteriologists and to which it is the object of this paper to call attention. These organisms grow better when a small amount of serum is added to media. Therefore, according to the custom of those who have been working with streptococci, a few drops of sterile horse serum were added to each tube of carbohydrate bouillon at the time the inoculation was made. Under these conditions acid was produced from dextrin and starch, whereas some of the simpler carbohydrates were not attacked. The possibility that this action might be due to enzymes in the serum which made the carbohydrates available to the bacteria was considered and the following experiments were made.

That various sera contain carbohydrate-splitting enzymes has been known to physiological chemists for years and the fact is stated in a number of text-books. Magendie (2) in 1846 found a starch-splitting enzyme in serum and Hamburger (3) showed that in addition to a diastase, a maltase was present. Moeckel and Rost (4) studied the sera of most of the domestic animals and found that they all contained a diastase. Invertase may be present in small amounts in some sera, though Abderhalden and Rathsmann (5) did not find it in dog serum until after they had injected saccharose into the circulation.

Among bacteriologists, Hiss specifically states that serum water should be heated to destroy the enzymes before the carbohydrate is added. Holman (6) found that streptococci grow better when unheated serum is used, and he introduced a medium containing 20 per cent of serum that is sterilized by filtration. Park and Williams (7) add an equal amount of sterile serum or ascitic fluid to a previously sterilized peptone water containing the carbohydrate to be used.

While the use of serum waters for the detection of acid is being gradually replaced by more accurate quantitative methods, the practice of adding a few drops of serum as an enriching substance is not at all uncommon. That this practice may give rise to misinterpretations is indicated by the following experiment.

Experiment 1.—Four fermentation tubes were prepared, one containing sugar-free bouillon, and the other three the same bouillon plus 1 per cent of Kahlbaum's soluble starch. Horse serum was added as indicated in Table I and the tubes were incubated over night to insure sterility. They were then inoculated from a bouillon culture of Morgan's Bacillus No. 1, an organism that attacks only the hexoses. After 5 days incubation the amount of gas was recorded and the amount of titratable acid was determined in the fluid of the bulb and of the branch of each

TABLE I.

Effect of Horse Serum on Starch.

Fermentation tube containing sugar-free bouillon plus.		Results of inoculation with Morgan's Bacillus No. 1 and incubation for 5 days.		
Starch.	Serum.	Gas.	Reaction of.	
			Bulb.	Branch.
per cent		*per cent*		
1	None.	None.	Neutral.	Acid, 1.3 per cent.
1	1 cc. = 4 per cent.	30	Acid, 2.3 per cent.	" 3.5 " "
1	2 " = 8 " "	23	" 3.7 " "	" 3.3 " "
None.	2 " = 8 " "	Bubble.	" 0.5 " "	" 1.3 " "

tube. The results obtained are given in Table I and show that in the presence of serum or starch alone gas and acid are not formed, whereas when both of these substances are present both gas and acid are produced.

Since in the above experiment the smallest amount of serum used was approximately 4 per cent, another experiment was made to determine the least amount of serum that would act on starch.

Experiment 2.—To tubes containing 4 cc. of sterile 1 per cent soluble starch was added 1 cc. of various dilutions of sterile horse serum. After 2 days incubation 0.5 cc. from each tube was transferred to a tube containing 5 cc. of Benedict's solution and the tube heated to boiling in a water bath. Tests for bacterial growth in the original tubes were also made. The results of this experiment, given in Table II, show that 1.2 per cent of serum will attack the starch to such a de-

gree that in 2 days there are substances present that will give marked reduction of Benedict's solution, and with as little as 0.3 per cent serum there is still evidence of an hydrolysis of the starch.

That these enzymes are not affected by keeping sera in the refrigerator for a considerable length of time is shown by the fact that the serum of a horse, a cow, and a pig, each $1\frac{1}{2}$ years old, when added to starch solution caused, after 2 days incubation, the production of substances that reduced Benedict's solution. Heating horse serum

TABLE II.

Amount of Serum Required to Produce Reducing Substances for Starch.

Tube.	4 cc. of 1 per cent starch plus 1 cc. of horse serum.			Result of transferring 0.5 cc. to 5 cc. of Benedict's solution and boiling.	Test for bacteria growth.
	Dilution.	Serum.			
		per cent			
A	Undiluted.	20		Marked reduction.	Negative.
B	1:2	10		" "	"
C	1:4	5		" "	"
D	1:8	2.5		Reduction.	"
E	1:16	1.2	Incubated	"	"
F	1:32	0.6	2 days.	Slight reduction.	"
G	1:64	0.3		" "	"
H	1 cc. of distilled water (control).			No reduction.	"
I	1 cc. of serum plus 4 cc. of distilled water (control).			" "	"

to 50°C. for 15 minutes did not destroy the enzymes, but serum heated to 53° for 1 hour or 60° for 15 minutes no longer formed reducing substances in starch solution.

The effect of serum on carbohydrates other than starch was tested bacteriologically as follows:

Experiment 3.—Tubes, each containing 9 cc. of fermented bouillon, were autoclaved, and to sets of four was added 1 cc. of previously autoclaved aqueous 10 per cent solution of a carbohydrate to a tube. Eleven of the more commonly used carbohydrates were available for the study. It was realized that some of them, such as the pentoses and alcohols, would probably not be changed by the serum. To each of two tubes of a set was added 0.5 cc. of sterile horse serum, and all the tubes

were incubated for 3 days. Two tubes from each set, one with and one without serum, were then inoculated from a bouillon culture of Morgan's Bacillus No. 1 and the other tubes from a culture of *B. dysenteriæ* Shiga; both of these attack only the hexoses. After a further incubation of 2 days the contents of each tube were titrated and the results given in Table III were obtained.

These results show that serum will change maltose and dextrin so that they give the same bacteriological reactions as dextrose. There is a slight breaking down of saccharose, but other tests have shown that it is of little importance, and while it is possible that other carbohydrates may be changed though not broken down

TABLE III.

Effect of Horse Serum on Various Carbohydrates.

Fermented bouillon plus 1 per cent.	Titratable acid after inoculation with.			
	Morgan's Bacillus No. 1.		*B. dysenteriæ* Shiga.	
	5 per cent serum.	No serum.	5 per cent serum.	No serum.
	per cent	*per cent*	*per cent*	*per cent*
Arabinose....................	1.0	0.8	1.1	1.1
Xylose.......................	1.0	1.0	0.9	No growth.
Mannitol....................	0.7	0.5	1.0	0.9
Dulcitol.....................	0.7	0.6	1.1	1.0
Lactose......................	0.8	0.8	1.0	0.9
Saccharose..................	1.7	0.7	1.3	1.0
Maltose......................	4.4	0.5	3.8	1.0
Raffinose....................	0.8	0.6	0.9	0.7
Inulin.......................	1.0	0.5	1.3	1.2
Dextrin.....................	3.0	0.8	3.9	0.9
Salicin......................	0.8	0.6	1.1	1.1
Distilled water..............	0.8	0.6	0.9	0.8

to a hexose, we have no evidence that this takes place. It is fortunate that inulin and salicin are not attacked, as these two carbohydrates have been used so much in differentiating streptococci.

Maltose, dextrin, and starch are, then, in the presence of unheated serum, of no value in differentiating bacteria. They may, however, be of great value when used without serum. Maltose has not been generally used, since it often gives the same results as dextrose. In separating the fowl typhoid from *Bacillus pullorum*, maltose is of great value and it also helps in classifying the dysentery bacilli.

CONCLUSIONS.

It has been shown that enzymes in serum will change maltose, dextrin, and starch so that they will react as dextrose in media. These enzymes are destroyed by heating to 60°C. for 15 minutes, but they are present in sera that have been refrigerated for as long as 18 months. The practice of using carbohydrate media containing unheated serum should be discouraged, and if it is used the possibility that the carbohydrate may be changed by the enzymes present must be considered.

The writer is indebted to Mr. Henry Hagens, of this laboratory, for valuable technical assistance.

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INHIBITORY ACTION OF PARATYPHOID BACILLI ON THE FERMENTATION OF LACTOSE BY BACILLUS COLI. I.

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In nature, organic matter is attacked by a large variety of bacteria and in its disintegration a number of species may take part. A knowledge of just how the species are associated in any given substrate or in what order they succeed one another in the process of decomposition might be of great value to the biochemist in supplying a point of approach hitherto neglected. There is evidence that there is some order in the mode of attack and that this order is conditioned by the nature of the medium and certain environmental factors, such as temperature, reaction, and oxygen supply. In the following pages one single detail of this general problem was examined under conditions which excluded all but two bacteria, one following the other.

If a fermentation tube containing fermented bouillon plus 1 per cent lactose be inoculated with some member of the paratyphoid group, the multiplication causes a heavy clouding or even turbidity in the open arm.

The growth in the closed arm depends on the degree to which the muscle sugar has been removed. If this is complete, no growth takes place. The clouding of the branch is therefore proportional to the amount of muscle sugar remaining. Titration shows little or no change of reaction in the closed arm, beyond what is due to a movement of fluid from open to closed arm. The fluid in the open arm becomes neutral and finally alkaline to phenolphthalein, the degree of alkalinity being a function of the multiplication which in turn is a function of the alkali-tolerance of the particular strain used. If after 4 to 6 days of incubation the same tube is reinoculated, this time with a true colon bacillus known to ferment lactose, one of two things may happen:

1. The resulting fermentation takes the usual course as if no prior inoculation had been made; *i.e.*, the production of acid and gas goes on quantitatively as in a fresh tube.

2. The second organism multiplies actively, clouds the closed branch as usual, but no gas is produced. Titration shows that the usual amount of acid has been produced.

In order to determine whether the particular type of second or superadded fermentation is constant for the same organism, numerous tests have been made during the past 1½ years, with the result that with the same combination of cultures the same result follows.

Furthermore the various types of paratyphoid bacilli which happened to be on hand in the laboratory were tested to see just what grouping would result if this kind of superadded fermentation were used as a basis of classification. The results of this latter test are in many respects suggestive.

All true hog-cholera bacilli left the lactose medium uninfluenced. The fermentation of the second organism proceeded as in a fresh unused medium. Among the hog-cholera bacilli were those of widely different ages. The oldest one had been isolated by one of us early in 1886[1] and was described at that time as a membrane-producing variant. The most recent strain had been isolated by Dr. TenBroeck at Camp Upton late in 1918 (No. XVI).

Among cultures not isolated from swine, *Bacillus icteroides* Sanarelli acted like a true hog-cholera bacillus. That this organism may have been a true hog-cholera bacillus is made highly probable by the experiments of Reed and Carroll.[2] They found a complete identity of the two types in morphological, cultural, pathogenic, and agglutinative characters. *Bacillus icteroides* also produced fatal enteritis associated with necrosis of the mucosa of the large intestine in young pigs. One of these experiments was so carefully controlled that the, at that time, still unknown filterable virus of hog-cholera could hardly have come into consideration as a cause of the lesions. Moreover the autopsy notes do not suggest the filterable virus.

[1] Smith, T., *3rd Ann. Rep. Bureau Animal Industry, U. S. Dept. Agric.*, 1886, 622.

[2] Reed, W., and Carroll, J., *J. Exp. Med.*, 1900–01, v, 215.

Of the cultures isolated from swine which failed to give the same reaction as the true hog-cholera bacillus was a non-motile organism isolated by Professor T. J. Burrill from the liver of a pig in 1897, and described briefly by one of us[3] in 1899. This strain could be differentiated from the true hog-cholera bacillus only through the absence of motility and a reduced virulence. *Bacillus coli* following this produced only a large bubble of gas in lactose bouillon.

Among the cultures tried which belong to the first group are Hog-cholera Nebraska (1886), Massachusetts (1895), Maryland (1898), Arkansas (1899), and Hog-cholera X, XI, XII, XIII, XIV, and XVI, each from a different outbreak; also *suipestifer* Voldagsen, and the non-gas-producing hog-cholera strain of M. Dorset. In several of the above strains mutants had appeared in the stock cultures. One of these produced a viscid growth, the other had lost the power of gas production in lactose. All these acted alike in not restraining gas production subsequently by *Bacillus coli*.

Among the paratyphoid and *enteriditis* strains in the collection the following came under the second group: paratyphoid strains recently isolated from four calves and one fetus by one of us; paratyphoid Strains 225, 232, 234, and 242; Schottmüller A and B; paratyphoid from a pigeon and a cow's brain, all received from other laboratories; Rat-typhus I, III, IV, and V; Swine-typhus I, II, III, IV, and V, Spermophile typhus I, Dog-typhus I, Guinea pig-typhus VII, VIII, Mouse-typhus I, II, *Bacillus enteriditis* 47, 204, Gaertner (Kral and Mt. Sinai), and swine *enteriditis*. The one exception noted thus far was marked paratyphoid (Longcope). Dr. TenBroeck[4] had previously examined this strain and found it culturally and serologically a hog-cholera bacillus. Its virulence, however, towards rabbits is very low as compared with true hog-cholera bacilli.

One culture of human typhoid tried thus far inhibited gas production and belongs therefore to the second group. A culture of paratyphoid bacilli from man which Hirschfeld has denominated Group C and which has been shown by TenBroeck[4] to belong culturally to the paratyphoid and serologically to the hog-cholera group, but with

[3] Smith, T., *Centr. Bakt., 1te Abt.*, 1899, xxv, 241.

[4] TenBroeck, C., *J. Exp. Med.*, 1920, xxxii, 33.

a reduced virulence, acted like a true paratyphoid in inhibiting gas production.

The rather sharp distinction which this reaction makes between true hog-cholera bacilli and other paratyphoid strains made it seem desirable to test the several members of the dysentery group on hand. All strains tested, including those marked Shiga, Flexner, Hiss Y, Flexner-Harris, and Strong, acted like the hog-cholera group in that the second or *Bacillus coli* fermentation was not inhibited as to gas production. Similarly the group of paratyphoid-like bacilli attacking poultry (fowl typhoid and white diarrhea) did not inhibit gas production by *Bacillus coli*.

So far, to avoid confusion, the second fermentation has been restricted to two colon strains isolated from a calf. The strain of *Bacillus coli* used most constantly in the various experiments was isolated in December, 1918, from the liver of a calf (No. 302) affected with pneumonia and septic complications. It possesses motility to a certain degree, ferments dextrose and lactose with the accumulation of from 50 to 60 per cent gas in the closed arm of the fermentation tube, and produces a titratable acidity of 4 to 6 per cent of a normal solution. The gas formula H/CO_2 is of the usual type, the explosive portion being 2 to $2\frac{1}{2}$ times the CO_2 in volume. Saccharose is fermented more slowly and about 25 per cent gas accumulates. The second strain (No. 435) was isolated from the spleen of a very young calf affected with digestive derangements, called scours. It was in all cultural characters like the preceding.

Wherever gas production is referred to in the following pages, the accumulation of gas in the fermentation tube is meant. The acid produced is quoted in per cent of titratable acid in terms of a normal solution.

It is to be predicted that all races of *Bacillus coli* will not act alike since there are so many minor distinguishing morphological, cultural, fermentative, and serological features observed among them. Some preliminary tests with other colon bacilli have been made but the data are incomplete.

Experiments to Determine the Nature of the Inhibition.

The Effect of Removing the Paratyphoid Bacilli.—The first suggestion to present itself was that the paratyphoid bacilli might have changed the lactose in some way. If so, in the bouillon deprived of the paratyphoid bacilli by filtration the colon bacilli should act differently than in fresh bouillon. It was found, however, that the complete removal of paratyphoid bacilli from the culture fluid by passing it through a Berkefeld filter restores gas production. The experiments were made by growing the paratyphoid bacilli in large centrifuge bottles and filtering after centrifuging. The filtrate was put into sterile fermentation tubes and inoculated with *Bacillus coli*. Table I gives some of the results.

<div align="center">TABLE I.</div>

Strain of paratyphoid used.	Age of culture when centrifuged and filtered.	Results of inoculation with *B. coli*, Calf 302.	
		Gas.	Acid in open arm.
	days	*per cent*	
Calf 299......................	10	26.5	5.10
" 299......................	10	45.0	4.75
" 297......................	6	47.2	4.60
" 297......................	6	52.0	

The next step was to proceed as in the former experiment but to omit the filtration. The centrifuged fluid did not become entirely clear and when inoculated with *Bacillus coli* only a very little gas was produced. Apparently the presence of a very faint cloud of paratyphoid bacilli was sufficient to inhibit almost completely gas production by *Bacillus coli*. The hog-cholera control treated in the same way did not inhibit gas production (Table II).

In order to remove as far as possible all bacteria without subjecting the culture fluid to final filtration, the following procedure was carried out. The primary culture was conducted as in the foregoing experiment in large centrifuge bottles, holding 250 cc. and plugged with cotton wool. After an incubation of about 4 days sufficient to yield a maximum multiplication a certain quantity of sterile kaolin powder was added to the fluid, the bottles were closed with

sterile rubber stoppers, and thoroughly shaken by hand. They were then centrifuged for about 30 minutes, at the end of which time the supernatant fluid was clear to the unaided eye in transmitted light. It was then transferred with sterile pipettes to sterile fermentation tubes and inoculated with *Bacillus coli*, Calf 302.

TABLE II.

Strain used.	Age of culture when treated.	Time of centrifugation.	Results of inoculation with *B. coli*, Calf 302.	
			Gas	Acid in open arm.
	days	*min.*	*per cent*	
Paratyphoid, Calf 297.........	7	30	Bubble only.	3.80
" " 297.........	7	30	1–2	4.61
Hog-cholera XII.............	10	30	60	5.30

To determine whether the kaolin itself might add anything to the medium to influence the result various controls were introduced as shown in Table III.

TABLE III.

Effect of Centrifuging and Clearing First Culture with Kaolin on Second Culture.

Experiment.	Treatment of first culture. (Paratyphoid, Calf 299.)	Results of inoculation of second culture. (*B. coli*, Calf 302.)	
		Gas.	Reaction of open arm (acidity).
		per cent	
a...........	Sterilized kaolin added, bottle shaken and centrifuged 30 min., transferred to fermentation tubes.	65	4.56
b...........	Sterile control fluid, treated as under a before inoculating with *B. coli*.	93	4.55
c...........	Sterile control fluid without kaolin.	62	5.40
d	Fermented bouillon without lactose, shaken with kaolin.	0	0.27 (Open arm.) 1.58 (Closed ")

Tube *a* shows that a complete clearing of the culture fluid from paratyphoid bacilli restores gas production by *Bacillus coli* and is equivalent to filtration. Tubes *b*, *c*, and *d* were to show whether kaolin modified the fermentation. The larger amount of gas produced in *b* indicates the presence of some favoring impurity on gas produc-

tion, possibly a carbonate. Tube *c* checked the culture medium and Tube *d* proved the absence of fermentescible sugars in the kaolin.

Effect of Density of Suspension of Paratyphoid Bacilli.—In the foregoing tests the primary culture was usually 4 days old before the second inoculation was made. The mere presence of paratyphoid bacilli in the culture fluid might have some influence irrespective of any incubation period. Experiments were therefore undertaken to eliminate this period. The growth of paratyphoid bacilli on slanted agar was washed off with bouillon and suspended in lactose bouillon in fermentation tubes. The suspension was varied in density by using the growth of one, two, and four slants respectively. Immediately after the suspension was made a loopful of *Bacillus coli* was introduced from an agar culture and the tubes incubated. The gas production was not inhibited but it accumulated more slowly than in control tubes. After 7 days incubation 70, 68, and 66 per cent of gas had accumulated. The reaction of fluid in the bulb was 4.61, 4.96, and 4.91 per cent of a normal acid respectively. It should be stated that the density of the suspension of paratyphoid bacilli, even in the closed arm of the first tube, was much greater than that developing directly in lactose bouillon. No inhibition of gas production was thus produced by the mere presence of large numbers of living paratyphoid bacilli, both strains being introduced together.

In another experiment, the relation of the bacilli to the inhibition was put to a more rigorous test. A large centrifuge bottle containing lactose bouillon and inoculated with paratyphoid, Calf 297, was incubated for 6 days. One fermentation tube was then filled with the culture fluid and inoculated with *Bacillus coli*, Calf 302, and incubated as a control. Another lot from the same bottle was centrifuged until the supernatant fluid was only faintly clouded. This fluid was transferred to a second fermentation tube and inoculated with *Bacillus coli*. The thick bacterial sediment in the centrifuge tubes from 30 cc. of the culture fluid was transferred to 25 cc. of fresh lactose bouillon in a fermentation tube and inoculated with *Bacillus coli*. Over 50 per cent gas was promptly produced in the third tube. In the others a small bubble appeared partly derived from fine bubbles transferred with the culture fluid.

The Effect of the Concentration of Lactose.—In the foregoing tests 1 per cent lactose was used throughout. It was thought, however, that less or more might affect the uniformity of results. Before taking up the experiment it became necessary to consider what minimum per cent of lactose was necessary for *Bacillus coli* to produce the maximum amount of gas. Earlier, repeated trials by one of us had shown that 0.5 per cent lactose yields about as much gas as 1 per cent or more. Table IV shows that the same result is obtained whether a concentration as low as 0.5 per cent or as high as 2 per cent lactose is used. Numerous tests of the gas formula in these experiments indicated no appreciable change in the relative amounts of H and CO_2. These tests have therefore been omitted from the tables.

TABLE IV.

Effect of Different Concentrations of Lactose on Secondary Fermentation by B. coli, Calf 302.

Primary culture.	Lactose.	Interval between primary and secondary cultures.	Gas produced.	Acid produced.	
				Open arm.	Closed arm.
	per cent	*days*	*per cent*		
Paratyphoid 297.....................	0.5	6	0	5.0	5.0
Hog-cholera XII.....................	0.5	6	57	5.2	
Paratyphoid 297.....................	2.0	4	0	3.7	4.8
Hog-cholera XII.....................	2.0	4	62	4.8	

The absence of lactose in the primary culture does not interfere with the establishment of inhibition. Cultures of paratyphoid bacilli in fermented bouillon without lactose will inhibit subsequent gas production by *Bacillus coli* when the latter and lactose are added at the same time after the primary culture has grown 4 or more days. To ensure the above results, there must be distinct clouding of the closed arm by the paratyphoid bacilli. Absence of multiplication due to a too thorough removal of muscle sugar fails to establish inhibition.

Effect of the Age of the Primary Culture.—Certain experiments had indicated that the age of the primary culture before inoculation with *Bacillus coli* was not an indifferent factor. Taking the combination

paratyphoid, Calf 297, and *Bacillus coli*, Calf 302, *Bacillus coli* was inoculated at the same time with and hours after the paratyphoid bacillus to determine how long a growth period is required by the primary culture before it becomes completely inhibitory.

Table V shows that inhibition begins after 4 hours and is nearly complete at 14 hours with one lot of bouillon. With another it is not complete at 72 hours. This difference is probably due to the degree of removal of the last traces of muscle sugar during the prep-

TABLE V.

Effect of Age of Primary Culture on Inhibition of Gas Production.

Age of primary culture before second inoculation.	Amount of gas produced.	Acid produced in open arm.	Acid produced in closed arm.
Bouillon 1,003.			
	per cent		
0 (Simultaneous.)	58	4.2	
2 hrs.	53	3.9	
4¼ "	28	3.9	4.0
6¼ "	24	4.2	3.9
8 "	20	3.8	4.2
10 "	11.5	4.4	4.2
12 "	10	4.9	4.2
14 "	5	4.6	4.9
Bouillon 985.			
21 hrs.	13	6.0	4.7
2 days.	12.5	4.9	4.7
3 "	10	5.3	3.8
4 "	0	4.2	3.9

aration of the fermented (lactose) bouillon which controls the clouding of the closed arm.

It has been observed in certain experiments in which the second organism was introduced later than 4 days after the first, that in those cultures in which gas production was not inhibited, as in the hog-cholera group, the total quantity of gas produced was nevertheless not up to the usual amount. This observation led to the inference that perhaps here also the time element was a determining factor.

Table VI gives the results of an experiment to clear up this question. Hog-cholera bacilli were introduced into a number of lactose tubes and kept different periods of time before *Bacillus coli* was introduced. Cultures incubated 19 days before the second inoculation inhibited gas production completely. Those incubated up to 6 days failed to inhibit. The rest produced variable amounts of gas.

The paratyphoid bacillus thus produces a condition in 2 to 4 days which the hog-cholera bacillus brings about in 18 days. It is interesting to note that this period roughly corresponds to the time

TABLE VI.

Effect of Age of Primary Culture on Inhibition of Gas Production. (Primary Culture, Hog-Cholera Bacillus XII; Secondary Culture, B. coli, Calf 302.)

Age of primary culture when *B. coli* inoculated.	Amount of gas produced.	Acid produced.	
		Open arm.	Closed arm.
	per cent		
0 (Control.)	65	5.0	4.9
3 days.	60	5.2	5.7
6 "	50	6.2	5.6
11 " *	5	5.9	5.6
11 "	32	4.6	5.2
14 "	7	4.8	5.5
18 "	0	4.8	6.0
19 " *	0	4.9	5.0

* Primary culture grown with about ⅓ of closed arm containing air. This was tipped out when *B. coli* was inoculated.

required by the hog-cholera bacillus to produce a translucency of milk when used as a culture medium. This translucency proceeds parallel with the increasing alkalinity of the medium. The different behavior of the paratyphoid and the hog-cholera bacilli is thus quantitative rather than qualitative, but the difference is such that at a certain time the phenomenon may be used as a qualitative test.

The gradual development of inhibition by the hog-cholera group is paralleled by a gradual loss of inhibition by the paratyphoid bacillus. This was demonstrated in the following manner. A culture of the paratyphoid bacillus, Calf 297, grown in lactose bouillon in a

centrifuge bottle was transferred to fermentation tubes after 7, 24, and 32 days and then inoculated with *Bacillus coli*, Calf 302. The 7 day culture inhibited gas formation completely, the 24 day culture developed 44 per cent gas, the 32 day culture 35 per cent. The three tubes developed the usual amount of acid. In another experiment a culture of paratyphoid bacilli 22 days old yielded after inoculating with *Bacillus coli* 13 per cent gas. It is probable that the inhibition produced after 18 days by the hog-cholera bacillus is gradually lost later on, but no experiments have been made to test this assumption.

The Effect of Killing Paratyphoid Bacilli by Heat on Inhibition of Gas Production by Bacillus coli.

The results of the various experiments made thus far led to a study of the behavior of dead bacilli on gas production. Numerous experiments were carried out with a variety of controls in each but the results were not entirely concordant and pointed to some neglected factor. In all cases the exposure to temperatures which failed to kill the first culture failed to destroy inhibition. After the thermal death point had been reached the results became irregular, but the experiments all agreed in that inhibition was destroyed as the temperature rose and at 100°C. and above gas production was more or less completely restored. A careful analysis of the details of the experiments which are not reproduced here led to the hypothesis that mere death of the first culture is not sufficient to destroy inhibition but that there is another factor involved which disappears rapidly on exposure to high temperatures or gradually at lower incubator temperatures. To demonstrate the gradual disappearance of inhibition the experiments given in Table VII were made. Cultures of paratyphoid bacilli in lactose bouillon contained in large centrifuge bottles were exposed to 62°C. for 35 minutes. Subcultures were made at once and after 1 or more days of incubation to determine whether any bacteria had survived. The culture fluid was transferred to fermentation tubes at once and after the heated fluid had been incubated for 1 or more days. Subcultures were made at each transfer to determine sterility.

TABLE VII.

Effect of Incubation of 5 Day Cultures of Paratyphoid Bacilli (Calf 297) Heated at 62°C. for 35 Minutes on B. coli (Calf 302).

No. of days in incubator.	Gas produced.	Acid in open arm.	Remarks.
	per cent		
0	6–7	4.87	Subculture sterile.
1	5	5.60	" "
3	60	5.10	" "
5	59	5.19	" "
0	4	4.97	" "
4	54	6.00	" "
7	56	5.18	" "

DISCUSSION AND SUMMARY.

Bacteria of the paratyphoid group may be divided into two classes according to the behavior of 4 day cultures in lactose bouillon after a second inoculation with certain types of *Bacillus coli*. One class includes all true hog-cholera bacilli, the other nearly all true paratyphoid and *enteriditis* types. Under the imposed conditions *Bacillus coli* produces the usual amount of gas in the presence of the first group. In the presence of the second no gas or only a bubble appears. The production of acid is not interfered with.

The significance of the inhibition was investigated in a variety of ways suggested by the particular hypothesis entertained at the time. Two main possibilities presented themselves; first, the direct association of the inhibition with living paratyphoid bacilli, and, second, the existence of a ferment or other product of growth as the inhibiting agent.

The theory that the living bacilli or those killed at the lowest possible temperature are responsible was favored by a number of experiments. Thus the complete removal of bacteria by filtration, or by centrifugation combined with the use of kaolin to produce a clear fluid restored gas production. The presence of a fine cloud of bacteria was sufficient to inhibit. On the other hand, the addition of large numbers of living bacteria from agar slants or from lactose bouillon after the requisite incubation period to fresh lactose bouillon

failed to inhibit gas production when *Bacillus coli* was added simultaneously.

When the inhibiting culture was heated at 62°C. for 35 minutes to sterilize it, gas production was still largely inhibited. But it was restored when higher temperatures were used, completely at 100°C. and above. It was also gradually restored by exposing the heated culture to 37°C. for 3 or more days.

The presence of variable amounts of lactose, or even the complete absence of lactose did not interfere with the development of the inhibitory factor.

The activity of the inhibition factor presents itself in the form of a curve, beginning at 0 when both paratyphoid and colon bacilli are inoculated simultaneously and rising as *Bacillus coli* is inoculated at longer intervals from the paratyphoid bacilli. The maximum of inhibition is reached at about the 4th day; thereafter it remains at the same level for a few days and then gradually falls until it is lost within 3 or 4 weeks. The curve of the hog-cholera group is delayed in that the maximum inhibition is reached at the end of 3 weeks. These curves have not been accurately determined. Taking into consideration all the accumulated data the writers tentatively present the hypothesis that the inhibitory factor is some metabolic product of the paratyphoid bacillus, possibly an enzyme, which is destroyed at a temperature somewhat above the thermal death point of the bacilli and which more gradually disappears from incubated cultures. The substance fails to pass Berkefeld filters. It is carried down mechanically with substances clearing the culture fluid.

The experiments support current theories which hold that the acid-producing and gas-producing entities in cultures are distinct.

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